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THE STABILITY OF BACTERIAL SUSPENSIONS.

I. A Convenient Cell for Microscopic Cataphoresis Experiments.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 10, 1922.)

Measurements of cataphoresis of particles in an electric field are complicated by the fact that in addition to the motion of the particles relative to the water, the water itself moves at the surface of the cell. In macroscopic measurements the cell is large enough so that the movement of the water as a whole is negligible. If the measurements are made in a narrow cell, however, the motion of the water is very great and must be corrected for. The theory and technique of such measurements have been thoroughly discussed by Ellis[1] and Powis.[2] As Ellis pointed out, the total motion of the water in a closed cell must be zero, since the water which moves one way at the surface of the glass must return in the opposite direction in the center of the cell. The *average* observed motion of the particles at all depths relative to the cell must, therefore, be the true motion of the particles relative to the water, which is the desired value. It is also necessary to use some form of non-polarizable electrodes in order to avoid the formation of gas bubbles. The cell devised by Powis answers the requirements but is troublesome to use if a large number of experiments are made. The cell shown in Fig. 1 has been found very convenient.

Construction of the Cell.—The cell itself is made of a thin slide resting on strips of glass about 0.8 mm. thick cemented to a thick glass slide. Two blocks of thick glass are cemented on top of the cover-slide at each end of the cell. The ends of the cell are then ground smooth on an emery wheel. A piece of thick walled glass tubing is widened and flattened at one end so as to cover the end opening of the

[1] Ellis, R., *Z. physik. Chem.*, 1911, lxxviii, 321; 1912, lxxx, 597.

[2] Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 91.

cell. This flattened end is ground smooth and is cemented to the end of the cell. The same process is repeated at the other end of the cell. The method of arranging the electrodes is apparent from the figure. The best material for cementing the cell together was found to be soft "De Khotinsky cement." The pieces of glass are warmed to about 80° in an air bath, coated with a thin layer of cement and pressed together. The surplus cement is then removed with wire and the last traces are wiped off with a piece of paper moistened with toluene. After the end-pieces have been fastened to the cell it is

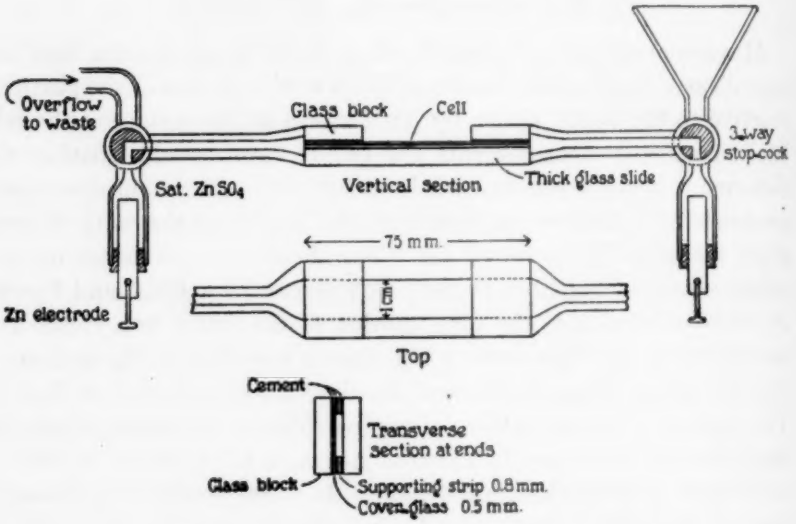

FIG. 1.

advisable to run more cement around the joint to strengthen it; this can easily be done by using a small soldering iron, the cement being handled in the same way as solder.

Calibration of the Cell.—It is necessary to know the drop in potential per cm. in the cell itself. Since the area of the connecting tubes, etc. in general is different from that of the cell it is necessary to correct for this difference. If the apparatus as a whole is filled with the same solution, as is the case during an experiment, the total resistance of the solution will evidently be proportional to the length and inversely proportional to the cross-section. Since the drop in potential per cm.

is proportional to the resistance per cm., it is possible to calculate the drop in potential in the cell itself, provided the dimensions of the rest of the apparatus are known. Expressed as an equation,

$$\text{Drop in potential per cm. in the cell} = \frac{\frac{L_c}{A_c}}{\frac{\frac{L_c}{A_c} + \frac{L_t}{A_t} + \frac{L_t'}{A_t'}}{L_c}} \times \text{total drop in potential}$$

L_c = length of cell in cm.
A_c = area of cross-section of cell.
L_t = length of tubing in cm.
A_t = area of cross-section of tubing.
The total potential is measured by a voltmeter connected to the Zn. electrodes.

In experiments with salt concentrations of less than tenth normal the resistance of the zinc sulfate is negligible. In any case the uncertainties due to the change in dielectric constant with increasing salt concentration are probably greater than the error introduced by neglecting the zinc sulfate. It can be seen from the above that failure to allow for a widening of the system at some point would make little or no difference in the potential gradient but that a narrow place in the system even though very short would cause a very large error. It is important, therefore, to be sure that the cell is not narrowed at the ends by the cement or by failure to align the ends of the cell and the side tubes.

Method of Measuring the Velocity of Migration.—The cell is clamped in position under the microscope, after filling the electrode tubes with saturated zinc sulfate, and the electrodes connected to a source of potential. The stop-cocks are turned so as to close the tubes containing the zinc sulfate and the cell filled with the suspension, care being taken to avoid air bubbles. The stop-cocks are then turned so as to connect them with the zinc sulfate solution, the circuit closed and the time required for a particle to cross a division of the micrometer eye-piece determined with a stop-watch. Owing to the migration of the water itself, it is necessary to obtain the average motion of the particles in the cell as a whole. This may be done accurately by determining the speed at various depths, say every 0.05 mm., plotting the curve so obtained and determining the mean height from the area

as measured by a planimeter and the length of the base. This is a time-consuming procedure and Ellis proposed a correction formula which required only two measurements, one at the surface of the cell and one at the center. This formula however, is based on the assumption that the velocity of the water in the center of the cell bears a constant relation to the velocity of the water at the walls of the cell. This ratio in turn depends upon the viscosity of the solution. The writer found that in the presence of proteins, serum, etc., the formula did not hold, owing presumably to changes in viscosity. It was found, however, that a value could be obtained which agreed with the true mean value by making measurements at the middle of each sixth or each eighth of the cell. Since the upper and lower halves of the cell are symmetrical, this requires either three or four measurements. It was found that the results were more reliable if a few measurements were taken at four depths than if the same total number of measurements were made at three depths. The procedure adopted was as follows: The apparent depth (on account of diffraction this is three-fourths of the actual depth) of the cell was 0.64 mm. as measured by the micrometer screw of the fine adjustment. The velocity of the particles was therefore measured at a distance of 0.04, 0.12, 0.20, and 0.28 mm. from the top of the cell, corresponding to the center of the first four eighths of the cell. Four measurements were made at each depth and the average of the reciprocals of these values taken as the true average velocity of the particles relative to the water. It is advisable to have a reversing switch in the circuit and take alternate measurements with the current reversed. These measurements should agree, and any divergence can usually be traced to air bubbles or a leak in the cell. When there is no potential across the cell the particles should remain stationary. Table I is an example of an experiment.

The potential is calculated from the observed velocity by means of the Helmholtz-Lamb equation as discussed in the preceding paper.[3]

Accuracy of the Method.—It was found in general that the measurements could be repeated within 1 to 2 millivolts. The calculated error from one series of measurements is considerably less than that, but the difference is probably due to the difficulty of making the measurements at exactly the correct depth. It is necessary, of course, to count

[3] Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921–22 iv, 635.

only those particles that are sharply in focus. This error could be lessened by using a deeper cell. It was found, however, that in a cell 2 mm. deep there was irregular drifting of the particles. Since the final value depends as a rule on the difference of two experimental values, the percentage error is larger the smaller the velocity.

TABLE I.

Rate of Migration of B. typhosus Suspension in Distilled Water.
Potential gradient = 4.5 volts per cm.

Distance from cover-glass at which determination was made.	Time to go 45 μ.	Average time.	Average μ per second.
mm.	*sec.*	*sec.*	*μ*
0.04	+15.0 +16.0* +15.5 +14.0	+15.2	+2.95
0.12	−4.5 −5.0 −4.0 −5.0	−4.6	−9.8
0.20	−2.5 −2.3 −2.4 −2.6	−2.45	−18.2
0.28	−2.0 −1.8 −2.0 −2.0	−2.0	−22.5
Average μ per second at all depths.....			−11.9
Average μ per second calculated for potential gradient of 1 volt per cm.			−2.65

Potential bacteria water, −33.5 millivolts.

* The sign refers to the apparent sign of charge of the particle, *i.e.* + indicates migration to the cathode.

Apparent Reversal of the Charge on the Glass.—It was noted that the direction of migration of the water reversed at times under the same conditions that caused a reversal in the motion of the particles. It is known that the sign of the charge on glass cannot be reversed so that this result is at first sight anomalous. The explanation is simple, however, since microscopic examination of the cell shows that it becomes more or less coated with the organisms, which adhere firmly to the glass. The cell wall is, therefore, no longer glass but is partially composed of the same material as the suspension and therefore reverses its charge under the same conditions as does the suspension.

AN APPARATUS FOR MACROSCOPIC CATAPHORESIS EXPERIMENTS.

By JOHN H. NORTHROP and GLENN E. CULLEN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 10, 1922.)

The form of apparatus[1] shown in Fig. 1 has been found very convenient for the determination of the migration of fine suspensions in the electric field. Its essential difference from the usual cataphoresis apparatus is that the whole is inverted so that the central portion, which contains the suspension which is to be studied, is above the heavier electrode solutions and may be left open. This increases both the convenience and accuracy of method since (1) a greater latitude in concentration of solution is allowed, (2) the boundaries may be adjusted more exactly, and (3) the solution may be renewed without disturbing the electrode solution. The zinc electrodes are put in place with rubber stoppers, the tube is clamped in a vertical position, and the apparatus is filled with saturated zinc sulfate. The three-way stop-cocks are then closed and the zinc sulfate in the upper part of the cell is washed out through the "tail holes." The tubes above the stop-cocks are now filled with 0.1 M sucrose solution containing the same concentration of electrolyte or other substance as is to be used with the suspension. The sugar solution is then allowed to run out until the level reaches the small tube connecting the funnel and the U-tube. The suspension is then added and the level adjusted carefully by means of the stop-cocks so that the line of demarcation is opposite one of the graduations on the side arms. The upper stop-cock is then closed and the lower ones are opened so as to connect the zinc sulfate with the sugar solutions. The current is applied and the distance traversed by the boundary determined after a convenient interval.

[1] For a discussion of this and similar methods see Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921.

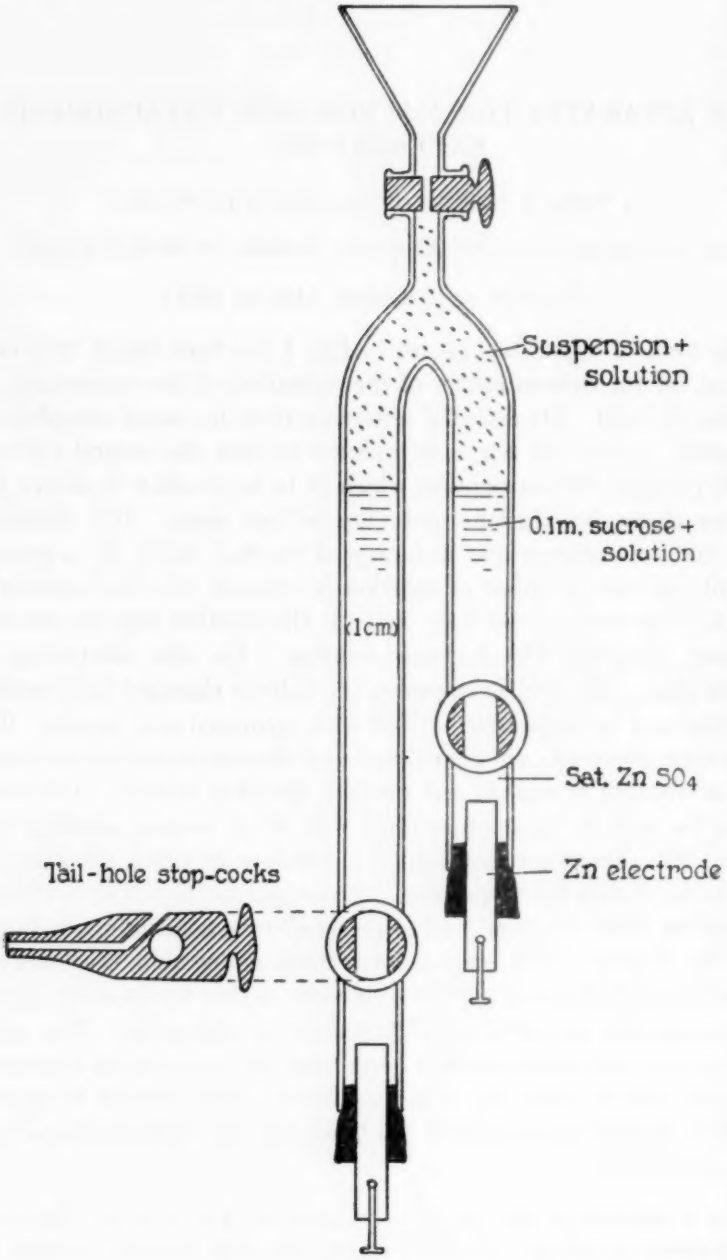
Suspension + solution
0.1m. sucrose + solution
(1cm)
Sat. Zn SO4
Zn electrode
Tail-hole stop-cocks

FIG. 1.

Since the cell is of uniform diameter the drop in potential is determined simply by dividing the total voltage by the distance between the three-way stop-cocks. (In solutions of less than 0.1 N the drop in potential in the saturated zinc sulfate may be considered as negligible; if more concentrated solutions are used it is necessary to apply a small correction for the resistance of the zinc sulfate.)

Influence of the Voltage.—It was found that the rate of migration was directly proportional to the voltage between the limits of 1 to 4 volts per cm., provided the experiment was not allowed to run too long. At low voltages, the rate of migration remained constant until the boundary approached the zinc sulfate, but if the potential drop was increased beyond 2 volts per cm., it was found that the boundary moved at the proper rate for the first 4 to 5 mm., but then became much too slow on one or both sides. There was also a tendency for the boundary to become convex on one side, showing that the migration of the water was interfering with the measurement. If the solution is of high conductivity, the voltage must be still further decreased to prevent heating effects and subsequent convection currents.

Influence of the Sugar Concentration.—The presence of sugar greatly facilitates the adjustment and maintenance of a sharp boundary line. No effect on the velocity of migration could be observed up to 0.5 M. Higher concentrations than this decrease the velocity presumably on account of the viscosity.

Calculation of the Potential from the Velocity of Migration.—The value for the potential difference between the particle and the surrounding solution is calculated by means of the Helmholtz-Lamb equation[1]

$$\text{P.D.} = \frac{4\,\eta\,v\,\pi}{K\,X}$$

in which

η = viscosity of the solution.
v = velocity of particle in cm. per second.
K = dielectric constant of the solution.
X = potential gradient; *i.e.*, the drop in potential in E.S.U. per cm.

All electrical units are electrostatic.

Substituting for the viscosity (0.009) and dielectric constant of water at 20° (80), and converting to volts it is found that P.D. in millivolts = 12.6 × μ per sec. × volts per cm. = 4.5 × mm. per hour × volts per cm.

In the experiments to be described no correction was made for a change in the dielectric constant nor viscosity.

The distance is taken as the average of the observed movement in the two arms. This corrects for any gravity effect. As a rule, however, the two readings are nearly identical and any marked discrepancy is apt to be the result of a leak in the apparatus or some other accidental error.

The apparatus has been found to work very satisfactorily for gelatin, edestin, the bacillus of rabbit septicemia, and other fine suspensions. With suspensions containing larger particles such as casein, typhoid bacilli, etc., the line of demarcation becomes blurred and the results are not satisfactory. For any particles that can be seen without an oil immersion lens the microscopic method is better.

Accuracy of the Measurements.—The measurements can usually be repeated as closely as can be read; *i.e.*, ± 0.1 mm. The error is greater in very dilute and very concentrated salt solutions.

THE STABILITY OF BACTERIAL SUSPENSIONS.

II. The Agglutination of the Bacillus of Rabbit Septicemia and of Bacillus typhosus by Electrolytes.

By JOHN H. NORTHROP and PAUL H. De KRUIF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 22, 1922.)

It is a very old observation that the stability of suspensions is markedly affected by the addition of electrolytes.[1] Under certain conditions the particles remain separate, while under other conditions they adhere to each other. In the latter case the particles settle rapidly and the suspension is said to be coagulated or agglutinated.[2] Since under certain conditions the particles remain distinct and in others collect into large masses, it is evident that there is a force which tends to hold them together and another force which tends to keep them apart. If the attractive force is greater than the repulsive force, the particles agglutinate. It was early found that nearly all substances in suspension are electrically charged with reference to the surrounding liquid, and it was suggested by Jevons that the repulsion due to this charge was the repelling force. This conception was substantiated by Hardy, who found that suspensions of denatured proteins coagulated at the point at which they carried no electric charge. Hardy called this the isoelectric point. Hardy's experiments have been greatly extended by Michaelis and his coworkers. It is probable, however, that the precipitation of proteins and the agglutina-

[1] For a review of the literature on this subject see Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921. In regard to proteins see Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

[2] The rapid settling of the coagulated suspension is a secondary phenomenon due to the increase in size of the particles and governed presumably by Stokes' law. The primary phenomenon is the adherence or repulsion of the individual particles.

tion of suspensions are governed by entirely distinct forces. In the case of oil emulsions Powis[3] was able to show that agglutination occurred whenever the potential became less than a certain critical value, in this case about 30 millivolts. Powis' experiments leave little doubt that the potential at the oil-water surface is the determining factor in the agglutination of oil emulsions. Burton[1] also found that metallic suspensions coagulate in the zone where the potential is small, although he did not find such a definite critical value.

In the case of bacteria, however, the results have been much less satisfactory. It was found by Bechhold,[4] Arkwright,[5] Teague and Buxton,[6] and others that bacteria were always negatively charged whether or not they were agglutinated. These authors concluded, therefore, that the potential carried by the organisms could not account for the phenomena. Putter[7] was able to show some qualitative agreement between the potential and agglutination of *Bacillus typhosus*.

Results of the Present Experiments.

It is evident that in order to test the hypothesis outlined above, it is necessary to measure both the force which tends to cause the particles to adhere as well as that which keeps them apart, since if both forces are affected by the conditions of the experiment but only one is measured, it will be impossible to interpret the results. The potential may be conveniently measured by the rate of migration in an electric field. The attractive forces, however, have usually been assumed to remain constant and no attempt has been made to measure them. It was found, in the course of the present experiments, that a comparative measure of the attractive forces between the organisms could be obtained by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution which was under investigation. As a result of

[3] Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 186.
[4] Bechhold, H., *Z. physik. Chem.*, 1904, xlviii, 385.
[5] Arkwright, J. A., *J. Hygiene*, 1914, xiv, 261.
[6] Teague, O., and Buxton, B. H., *Z. physik. Chem.*, 1907, lvii, 76.
[7] Putter, E., *Z. Immunitätsforsch., 1 te Abt., Orig.*, 1921, xxxii, 538.

these measurements in conjunction with the measurements of the potential difference, it has been found that whenever the potential difference between the surface of the bacteria and the solution is less than about 15 millivolts the bacteria agglutinate, *provided the cohesive force is not affected.* If the cohesive force is decreased, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 N affect primarily the potential, while in concentrations greater than 0.1 N the effect is principally on the cohesive force. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurement of the potential.

Experimental Methods.

Measurement of the Potential.—The potential was determined from the rate of migration as described in the preceding papers.[8] The U-tube method was used for the experiments with the bacillus of rabbit septicemia and the microscopic method with *B. typhosus.*

Measurement of the Cohesive Force.—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed organisms (*B. typhosus*), the film allowed to dry and then heated to 60° for a few minutes. This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the du Noüy[9] surface tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or

[8] Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921–22, iv, 635. Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[9] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.

fifteen measurements due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly reproducible. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as "cohesive" without an exact definition of its nature.

Measurement and Regulation of the Hydrogen Ion Concentration.—The pH determinations were made electrometrically, using a saturated calomel cell and taking the pH value of 0.10 N HCl as 1.04 at 33° as the standard.

Buffers Used.—It was found that a very convenient buffer could be made by combining sodium phosphate, sodium acetate, and glycine. It may be used over a range of pH from 1 to 13 and has the further advantage that the nature of ions present is not varied. The only variation is a change in concentration of the Cl and Na ions. The composition and the titration curve of the buffer are given in Fig. 1. This is referred to as G. P. A. Buffer. The pH measurements were made at 33°. In some experiments Walpole's[10] acetate series was used.

Cultures.—The culture of the bacillus of rabbit septicemia used was that previously isolated and described by one of the writers.[11] The typhoid culture was the Pfeiffer strain obtained through the kindness of Dr. Charles Krumwiede to whom we are also indebted for the strong antityphoid horse serum.

Measurement of the Degree of Agglutination.—No satisfactory method could be found for measuring the agglutination quantitatively. Several degrees of agglutination were, therefore, selected and the determinations made on this basis. They were recorded as follows:

− No agglutination.

+ Distinct particles visible with a lens, 8 diameters magnification.

++ Particles visible with the eye alone.

+++ Suspension almost completely agglutinated and settled but cloudy appearance in the supernatant liquid.

C. Supernatant liquid perfectly clear.

The stage marked C. is the easiest to detect with certainty and was used as the end-point.

The degree of agglutination increases with time at first but after 24 hours remains constant. All readings were therefore made after 24 hours at 20° to elimi-

[10] Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 2501.

[11] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

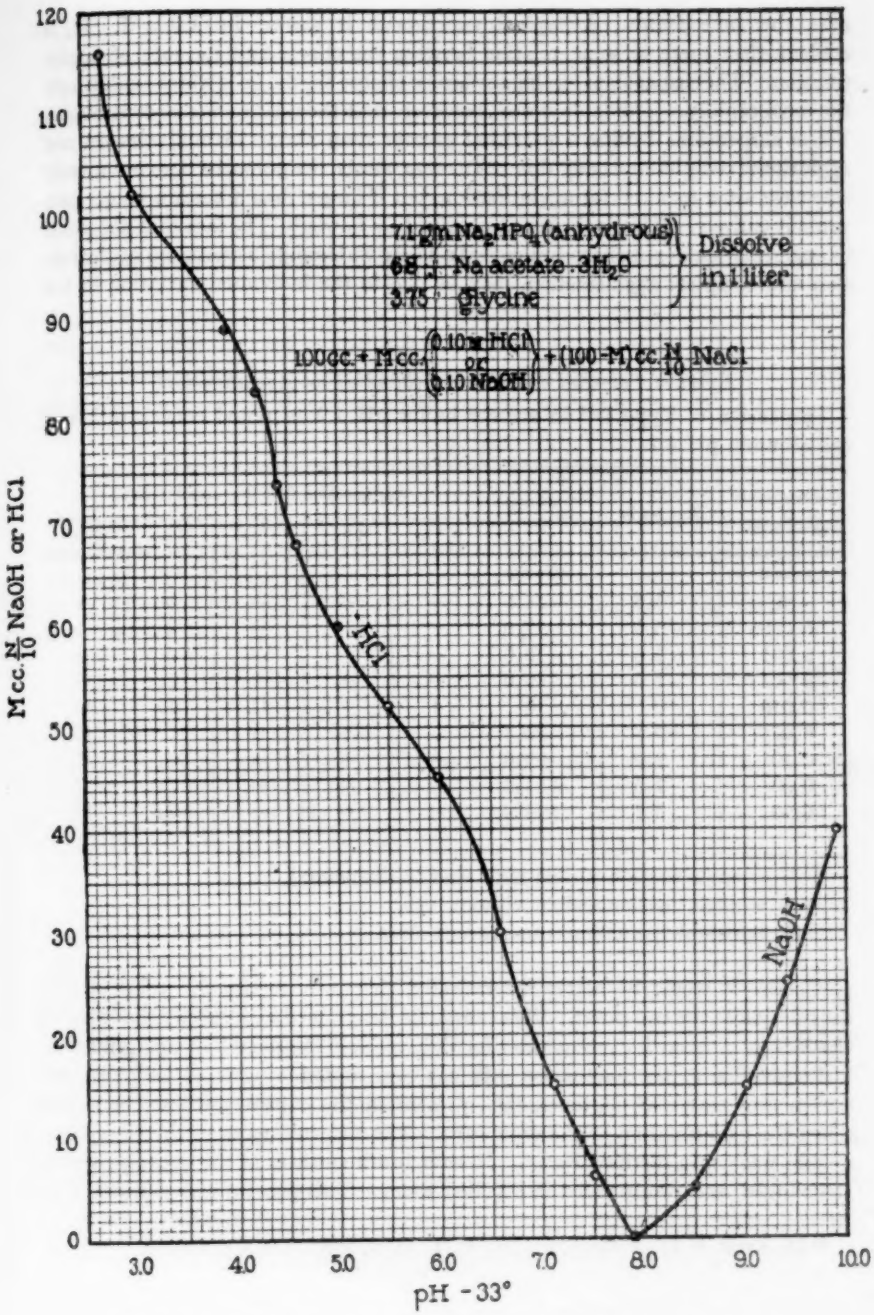

FIG. 1. Titration curve of glycine acetate phosphate buffer.

nate the time factor. A typical experiment is shown in Table I. It is evident that there is some relation between the charge and the rate of agglutination. The suspensions having the lowest charge are the ones which agglutinate the most rapidly. The table shows, however, that the relation is not continuous. Those suspensions having a potential greater than about 15 millivolts do not agglutinate completely at any time. In other words, the potential does not merely effect the time required for agglutination, but if larger than a certain value, prevents it entirely. This is the result obtained by Powis.[3] The fact that the point of agglutination is not sharp but covers a fairly wide range between no agglutination and complete agglutination, may be due to the individual variation in the

TABLE I.

Effect of Potential and Time of Standing on Agglutination.

Suspension of Type D[11] in acetate buffer pH 4.2 + noted concentration of egg albumin pH 4.2.

Concentration of egg albumin.	Mm. per hr.	Potential difference.	Agglutination after time noted at 20°C.				
			0.5 hr.	1 hr.	4 hrs.	24 hrs.	48 hrs.
per cent		*millivolts*					
0	−7.5	−34.0	−	−	−	−	−
0.0003	−6.0	−27.0	−	−	−	+	+
0.001	−4.0	−18.0	−	−	+	++	++
0.003	−2.0	−9.0	−	+	++	C.	C.
0.010	0	0	+	++	C.	C.	C.
0.03	+0.8	+3.4	−	++	++	C.	C.
0.10	2.0	+9.0	−	+	++	C.	C.
0.30	2.5	+11.2	−	−	+	C.	C.
0.90	3.2	+14.4	−	−	−	C.	C.

\+ = agglutination visible with lens (8 diameters).
++ = agglutination visible without lens.
C. = complete settling, supernatant clear.

particles. It would be better theoretically, therefore, to use the point of half coagulation as the end-point. This cannot be determined experimentally owing to the lack of a quantitative method for determining the degree of agglutination.

Preparation of the Suspension.—It has been noted by one of us[12] that the presence of traces of peptones, etc., present in the culture medium, markedly affect the agglutination of the organisms. The suspensions were therefore thoroughly washed in distilled water. 24 hour broth cultures of the organisms were centrifuged, and resuspended in distilled water. This process was repeated four

[12] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 395. See also Putter.[7]

times. The sediment finally obtained was then suspended in a volume of distilled water equal to one-half that of the original broth. For the determinations one volume of this "standard" suspension was added to one or two volumes of the other solutions used. Table II shows that no noticeable change could be detected after the second washing.

Effect of the Manner of Mixing and Time of Standing on the Potential.—No difference could be detected in the results obtained when the suspension was added to the solution or *vice versa*, provided the mixing was rapid and complete. As a rule the suspension was squirted into the solution from a pipette and mixed as thoroughly and rapidly as possible. No significant changes occurred in the potential measurements over an interval of 2 days except in the case of silver salts. The effect on the potential is, therefore, almost instantaneous in most cases. This is also true of the effect of immune serum, and shows that the time element consists in the time required for the organisms to come into contact.[13] In

TABLE II.

Effect of Washing on Rate of Migration of Type D Suspension.

100 cc. of broth culture, Type D, were centrifuged, suspended in distilled H_2O, centrifuged, and the process was repeated as noted. Migration was determined as noted.

No. of times washed	0	1	2	3	4
Potential at pH 4.4	−9.0	−18.0	−27.0	−28.0	−28.0
" " pH 3.0	−13.0	+1.6	+1.6		+1.8

the case of suspensions treated with silver salts at a pH of 4 or more, the potential drops rapidly and is very much lower after 24 hours. At the same time the suspension turns black so that the effect is probably due to the reduction of the silver.

EXPERIMENTAL RESULTS.

The results of the experiments are shown graphically in Figs. 2 to 8. The calculated potential in millivolts between the surface of the organism and the surrounding liquid is plotted as ordinates, and the salt concentration as abscissae. Since there is some doubt as to the correctness of the formula connecting velocities to millivolts, the actual velocities corrected for a potential drop of 1 volt per centimeter have also been given. The degree of agglutination is indicated by the character of the line. In the experiments in which no pH value

[13] This conclusion had been reached by F. L. Gates (*J. Exp. Med.*, 1922, xxxv, 63) in a study of the time required for adsorption of immune body.

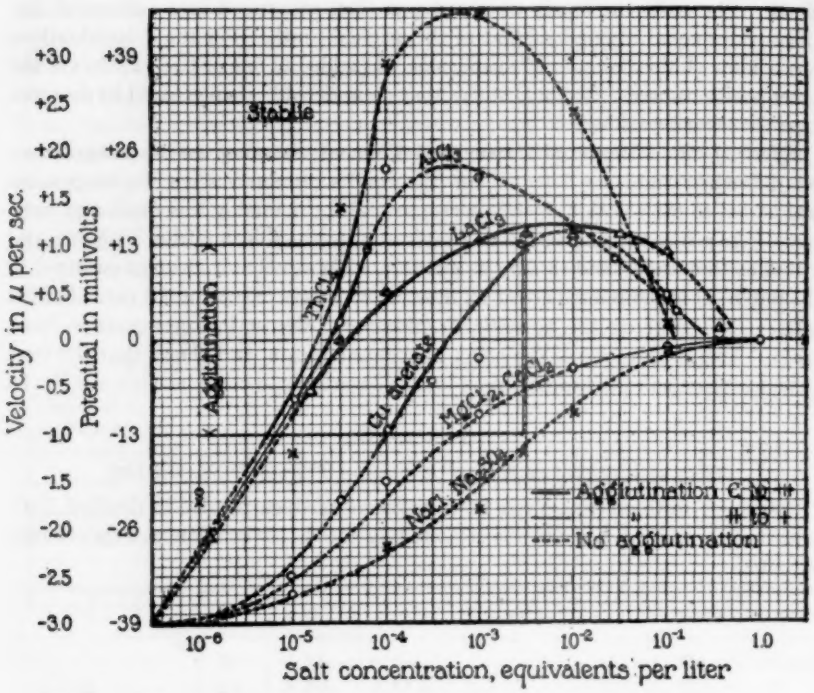

FIG. 2. Effect of salts on the potential and agglutination of *B. typhosus*.

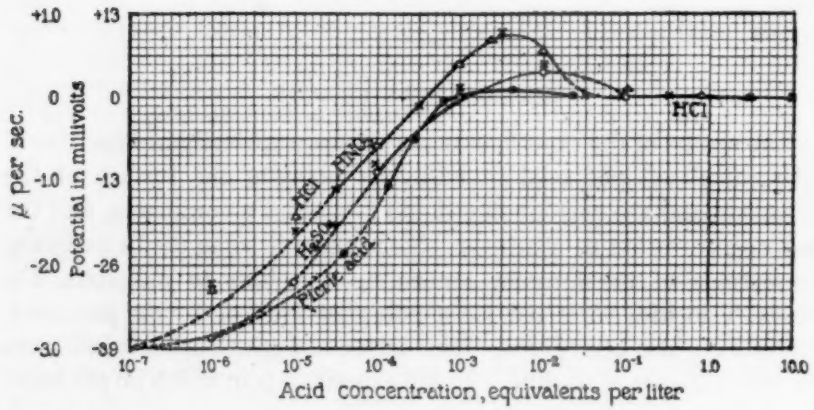

FIG. 3. Effect of acids on the potential and agglutination of *B. typhosus*.

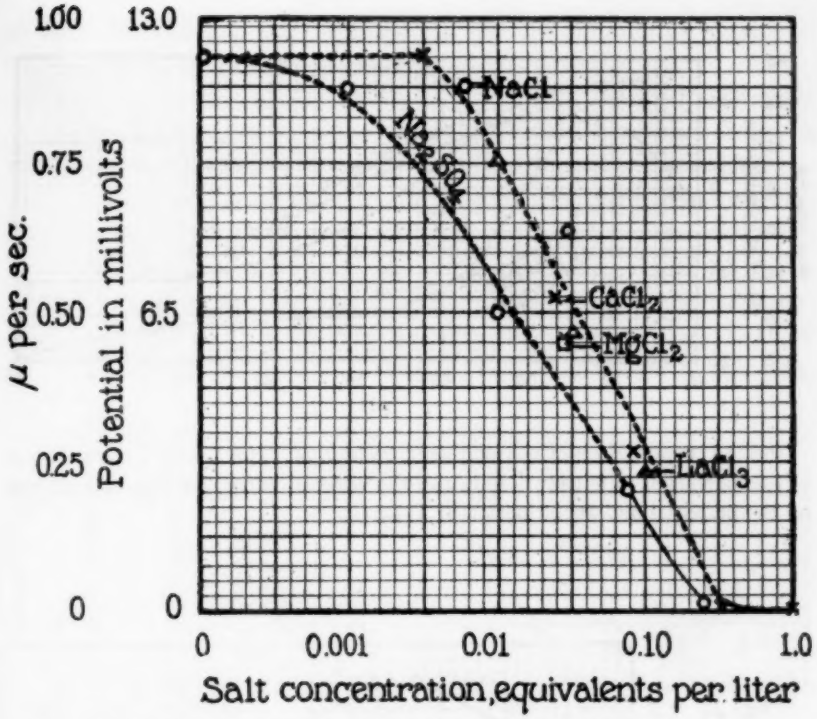

FIG. 4. Effect of salts on the potential and agglutination of *B. typhosus* at pH 2 (0.01 N HCl).

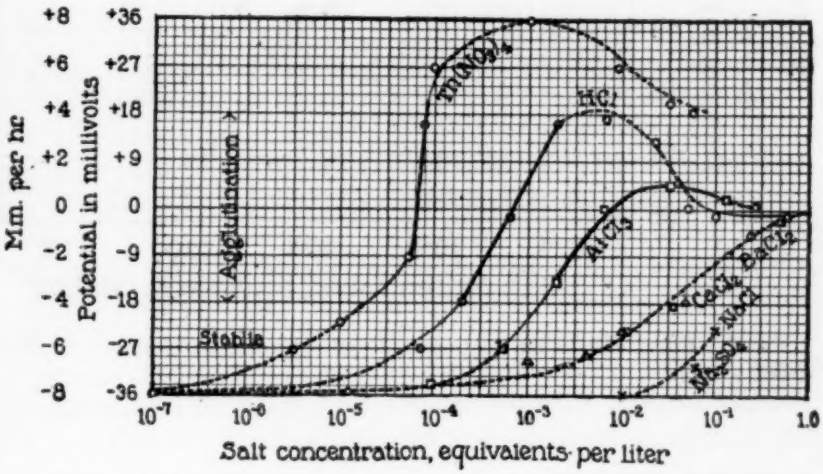

FIG. 5. Effect of salts on the potential and agglutination of the bacillus of rabbit septicemia Type D strain.

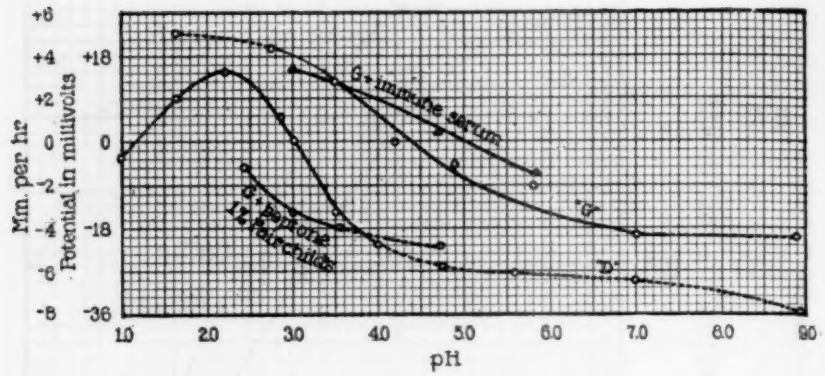

FIG. 6. Comparison of the acid agglutination of Type D and Type G strains of the bacillus of rabbit septicemia and the effect of immune serum and peptone on the potential and agglutination of Type G.

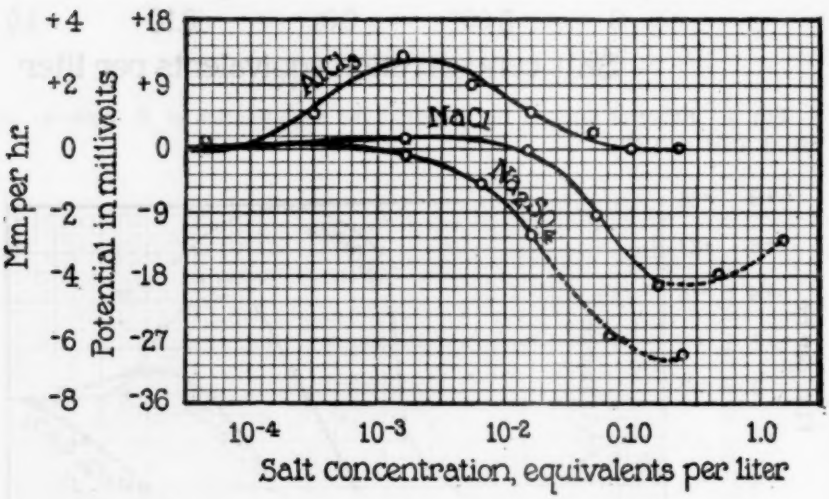

FIG. 7. Effect of salts on the potential and agglutination of Type D at pH 3 (0.001 N HCl).

is given, the pH was not regulated and the results are due in part to changes in the hydrogen ion concentration.

Inspection of the charts shows that in all experiments there is complete agglutination as soon as the potential is reduced below a value of about 15 millivolts (either positive or negative) provided the salt concentration is below 0.001 N. Below this salt concentration, therefore, the agglutination is seen to depend solely on the potential. Any substance which reduces the potential below about 15 millivolts will cause agglutination. There is another range of salt concentration above 0.10 N in which no agglutination occurs, although there is no measurable potential. Between these two ranges of salt con-

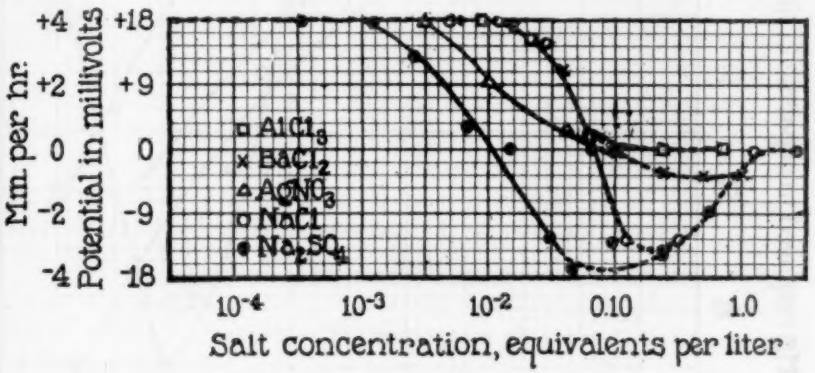

FIG. 8. Effect of salts on the potential and agglutination of Type D at pH 2.0.

centration there is a zone in which agglutination occurs at various potential levels. This is evidently the result that we would expect if the salt acted in low concentration primarily on the potential, and in high concentration on the cohesive force. There would be an intermediate zone in which the agglutination could not be predicted from either measurement alone. This explanation is borne out by the measurements of the cohesive force shown in Fig. 9. These show that the cohesive force is markedly decreased in concentrations of more than 0.01 N; *i.e.*, the range in which the critical potential begins to decrease. The figure shows that the effect on the cohesion is not connected with the valency nor with the electrical effects of the ions. $LaCl_3$ is far more effective than NaCl in reducing the potential, but

less effective in reducing the cohesive force. The agglutination depends on both factors. It is possible, therefore, for all monovalent ions to affect the potential in the same way but to differ in their coagulating power. In order to predict the coagulating efficiency of a salt, it is therefore necessary to know the effect on both the potential and cohesion.

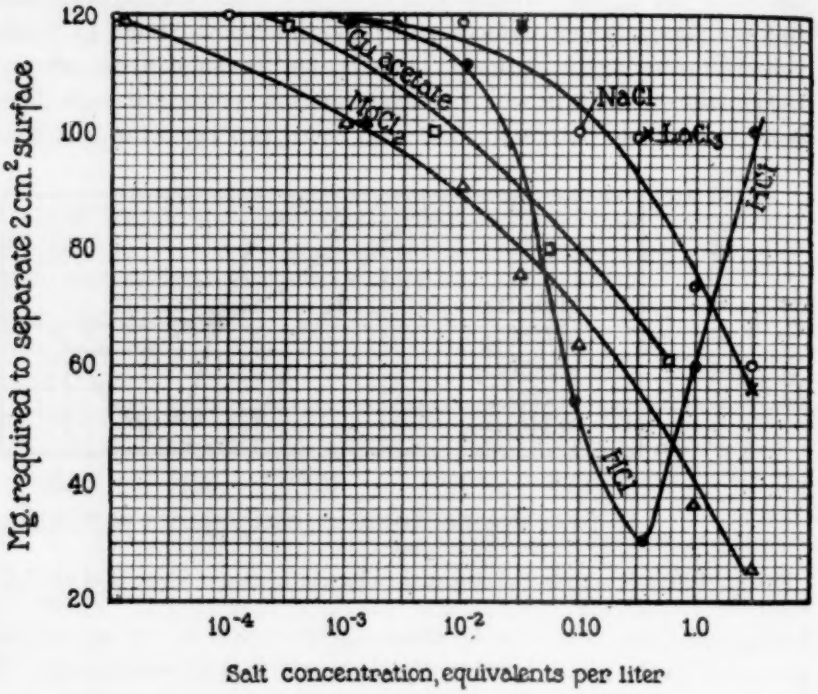

FIG. 9. Effect of salts and acids on the cohesive force between films of *B. typhosus*.

The HCl curve differs from the others in that the cohesion is *increased* in solutions of higher concentration than 0.3 N. This agrees with the agglutination test (Fig. 3) which shows a zone of agglutination at this concentration.

The experiments show the result usually obtained in such cases, that low concentrations of salt precipitate and higher concentrations stabilize again. They also show that this is due in most cases to the

fact that excess salt or acid confers a high potential upon the particles, of opposite sign to that in low concentration.

These various effects are all shown in the case of thorium chloride (Fig. 2). In concentration below 5×10^{-6} N no agglutination takes place since the potential is greater than 15 millivolts (the organisms being negative to the water) and the organisms are kept apart by the repulsion due to this potential. In concentrations between 5×10^{-6} and 5×10^{-5} N there is agglutination, since in this range the potential is less than 15 millivolts and the repulsion is therefore not sufficient to overcome the cohesion. In concentrations of from 5×10^{-5} to 5×10^{-1} the potential is greater than 15 millivolts (though of the opposite sign) and the suspension is again stable. At a concentration above 0.05 N the potential drops below 15 millivolts but agglutination does not occur since the cohesive force has also been reduced. A smaller potential is therefore sufficient to prevent agglutination. At a concentration of 0.10 N the potential is reduced practically to zero and agglutination again occurs. In still higher concentration the organisms are again stable due to a further decrease in the cohesive force.[14] The hydrochloric acid curve is interesting in that it shows a zone of agglutination in concentrated solutions (> 0.3 N). This is due to the sudden increase in the cohesive force at this point as is shown in Fig. 9. This does not occur with the other chlorides and in the latter solutions no agglutination occurs in this range.

The stabilizing effect of sodium chloride in high concentration is shown more strikingly in Fig. 10,[15] which gives the result of adding increasing salt on the acid agglutination zone. The addition of

[14] According to O. Porges (*Centr. Bakt., 1 te Abt., Orig.*, 1906, xl, 133) agglutination occurs again in very strong salt solutions such as half saturated $(NH_4)_2SO_4$. This is probably a salting out phenomenon, due to a decrease in the forces between the surface of the particle and the liquid. For a review of the effect of salts on agglutination see Buchanan, R. E., *J. Bact.*, 1919, iv, 82. The experiment itself shows that this is a different phenomenon since in saturated $(NH_4)_2SO_4$ agglutination occurs immediately whereas the type of agglutination studied in this paper requires considerable time.

[15] It will be noted that in this experiment the isoelectric point was about pH 4.2 while in others with *B. typhosus* (Fig. 3) it is about 3.5. This difference was noted several times and depends probably on the age and condition of the suspension.

0.01 N salt decreases the potential and broadens the agglutination zone slightly. More concentrated salts, however, although it reduces the potential still more, *decreases* the agglutination, since the cohesive force is now being reduced. In concentrations of more than 1.0 N no agglutination occurs. The salt also shifts the zone of agglutination to the acid side. This result has been obtained by Michaelis and Rona[16] with proteins.

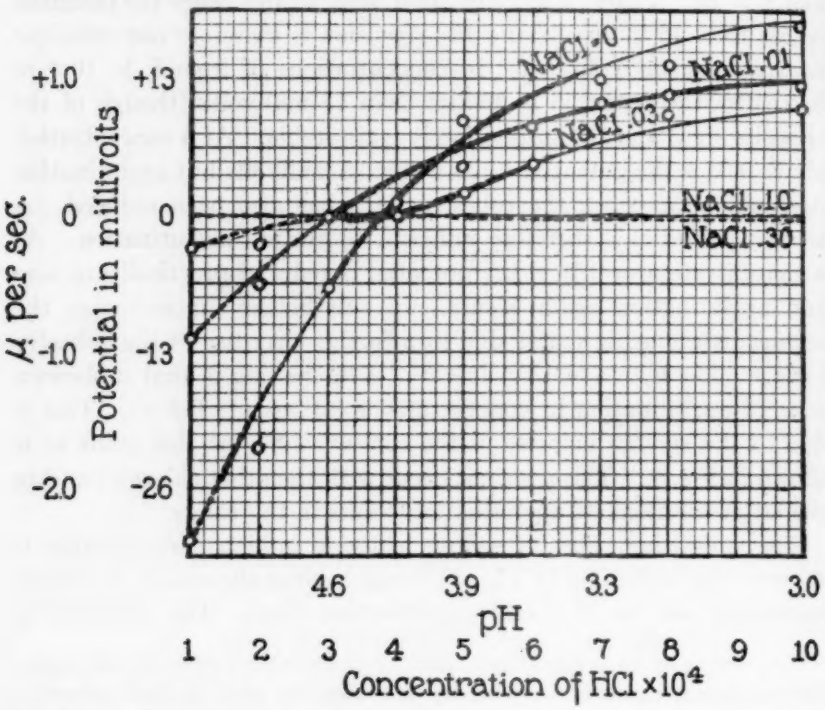

FIG. 10. Effect of NaCl concentration on the potential and agglutination of *B. typhosus* at the acid agglutination zone.

Effect of the Salts on the Potential.—The experiments show the familiar result that the effect is due to the oppositely charged ion and increases in general with the valence of the ion. The effect is not purely due to the valence since the hydrogen ion is far more active than the other monovalent ions. The result also depends on the nature of the

[16] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

suspension since the charge on the bacillus of rabbit septicemia may be reversed by sulfate or NaCl while with *Bacillus typhosus* suspension the charge is reduced but does not change in sign.

The experiments in Fig. 6 show clearly the reason for the characteristic difference in the stability of Types D and G of the rabbit septicemia bacillus.[11] Type D which is very stable has a high potential whereas the potential of Type G is very little more than the critical.

The same figure shows that the acid agglutination zone may be shifted markedly by the addition of other substances. Peptone for instance moves it far to the acid side (*cf.* Putter[7]) while immune body brings the isoelectric point to nearly 5. This point will be discussed more fully in the succeeding paper.

Origin of the Potential.—Loeb has shown,[17] in the case of a protein solution separated from a solution of electrolyte by a collodion membrane, that the charge on the protein solution can be quantitatively accounted for on the basis of Donnan's theory of membrane potentials. According to this theory, electrolytes affect the potential of a particle in two ways. (1) By combining chemically with the particle (for example hydrogen ions). The ion then becomes part of the molecule of which the particle (membrane) is composed. As a result the concentration of this ion differs on the opposite sides of the membrane and gives rise to a potential. This potential may be calculated by Nernst's formula from the concentration of the common ion on both sides of the membrane. The membrane behaves as a reversible electrode for this ion. (2) Ions which affect the distribution of the common ion without further chemical combination with the membrane. This mechanism will suffice to account for all the observations made in the course of this work, if it be supposed that other ions than the hydrogen ion may act by chemical combination.[18] The experiments are more complicated than those with a collodion membrane since the organisms are apparently more or less impermeable to ions.[19]

[17] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351; Proteins and the theory of colloidal behavior, New York and London, 1922, 120.

[18] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 164, 165; *J. Gen. Physiol.*, 1921–22, iv, 463; also two papers in this number of the *Journal* which the writer has had the privilege of reading in manuscript form (*J. Gen. Physiol.*, 1921–22, iv, 741, 759).

[19] Shearer, C., *Proc. Cambridge Phil. Soc.*, 1916–19, xix, 263.

SUMMARY.

1. Measurements have been made of the potential and of the cohesive force at the surface of *Bacillus typhosus* and the bacillus of rabbit septicemia in solutions of various salts and acids.

2. Electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration decrease the cohesive force.

3. As long as the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 15 millivolts.

4. When the cohesive force is decreased the critical potential is also decreased, and in concentrated salt solution no agglutination occurs even though there is no measurable potential.

THE STABILITY OF BACTERIAL SUSPENSIONS.

III. Agglutination in the Presence of Proteins, Normal Serum, and Immune Serum.

By JOHN H. NORTHROP and PAUL H. De KRUIF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 24, 1922.)

It has frequently been noted that the addition of a small amount of certain substances, especially proteins, markedly affects the behavior of suspensions. It was found by Whitney and Blake[1] for instance that the sign of the charge of gold particles in the presence of gelatin could be reversed by acids, a result which did not occur without gelatin. The same effect has been noted by Loeb[2] in the case of collodion membranes treated with different proteins. The membrane always acquires the isoelectric point of the protein used. It was found by one of the writers that peptone markedly affects the acid agglutination of the bacillus of rabbit septicemia. It has been shown in the preceding paper[3] that the isoelectric point was also displaced. This result had been noted by Putter.[4] The present paper contains the results of experiments on the effect of proteins and sera on the properties of suspensions of bacteria.

Fig. 1 shows the effect of various concentrations of egg albumin on the agglutination and charge of the bacillus of rabbit septicemia (Type D strain). The method of plotting is the same as in the preceding paper. Increasing the amount of egg albumin gradually shifts the curve to the alkaline side so that the isoelectric point is moved to pH 5.0 which is approximately that of egg albumin. In other

[1] Whitney, W. R., and Blake, J. C., *J. Am. Chem. Soc.*, 1904, xxvi, 1339.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 659; 1921–22, iv, 213.

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[4] Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538. The same observation had been made independently by one of the writers De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 345.

words, the particles act more and more like particles of egg albumin. The form of the curve is very similar to the curve found by Loeb[5] for the potential between a solution of egg albumin in a collodion sac and the surrounding solution. As was found in the experiments described in the preceding paper, agglutination occurs whenever the potential is reduced below a value of about 15 millivolts. The result of the addition of egg albumin is, therefore, that the agglutination zone is moved to the alkaline side and that at a pH of 3 the egg albumin stabilizes the suspension instead of precipitating it.

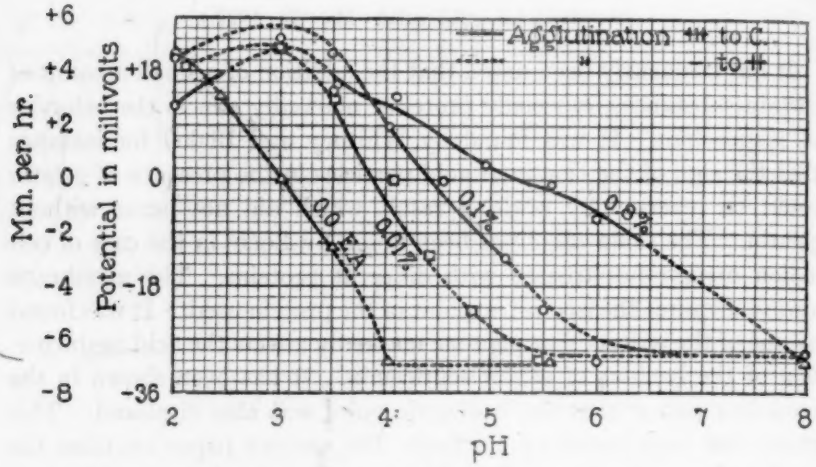

FIG. 1. Effect of the concentration of egg albumin at different pH on the potential and agglutination of Type D. pH adjusted with acetate buffers.

This is typical of the action of protective colloids and is due, as the figure shows, to the increase in the potential. The figure also shows that the amount of egg albumin required to agglutinate is a minimum near the isoelectric point of the suspension and increases as the pH is moved to the alkaline side. Similar experiments have been published by Eggerth and Bellows.[6]

Fig. 2 shows the effect of the addition of globin to a suspension of Type D; the isoelectric point is now shifted to pH 6.5 which is near

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[6] Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921–22, iv, 669.

the isoelectric point of globin. Agglutination again occurs whenever the potential is less than 15 millivolts.

The effect of normal and immune serum on the pH curves of *Bacillus typhosus* is shown in Fig. 3.[7] The result is very similar to egg albumin. It will be noted that there is no marked difference between the immune and the normal serum and also that the isoelectric point is shifted to a pH of 4.7 in both cases. This was an unexpected result, since the isoelectric point of blood globulin is given by Mi-

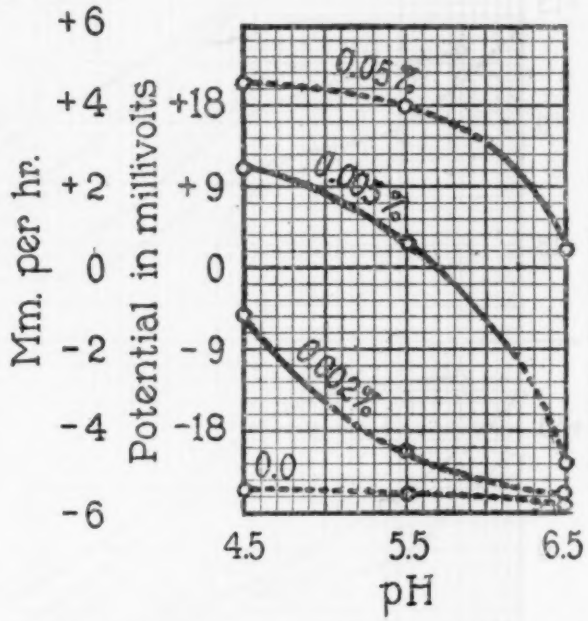

FIG. 2. Effect of the concentration of globin on the potential and agglutination of Type D at different pH. Acetate buffers.

chaelis[8] as 5.4 and the antibodies are known to be associated with the globulins. According to the present experiment, however, the substance in the serum which has the greatest effect on the charge of the

[7] Similar experiments have been made by Kōsaka and M. Seki, Communication to the Okayama Medical Society, *Okayama-Igakkwai Zasshi*, 1922, No. 386.

[8] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914. It is doubtful if this can be considered the isoelectric point of pure immune body since traces of foreign proteins have such a marked effect.

organisms has an isoelectric point at about pH 4.7. The small difference in the concentration of normal and immune serum required to change the isoelectric point renders it improbable that this effect can be ascribed to the immune body.

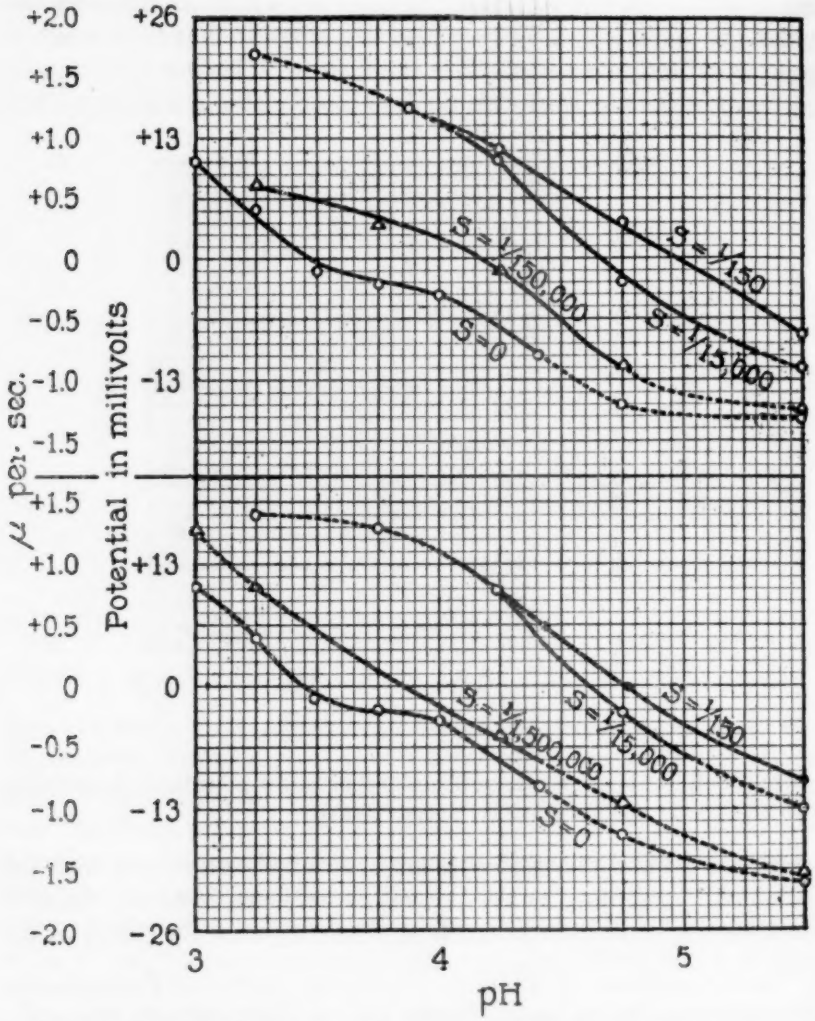

FIG. 3. Effect of different concentrations of normal (upper half) and immune serum (lower half) on the potential and agglutination of *B. typhosus* at different pH 0.01 N acetate buffer.

The stabilizing effect of the serum at pH 3 is likewise due to some constitutent of the serum other than the immune body as is shown by the following experiment. A suspension of *Bacillus typhosus* was treated with an excess of immune serum in 0.10 N salt and then washed once with distilled water. The suspension was then added to

TABLE I.

Agglutination of B. typhosus by Antityphoid Horse Serum at Various CH. *G. P. A. Buffer.*

pH	Concentration of serum. 5×10^{-4}	2.5×10^{-4}	1.25×10^{-4}	6.2×10^{-5}	3.1×10^{-5}	1.55×10^{-5}	7.8×10^{-6}	3.9×10^{-6}	Control. No serum.
8.5	C.	C.	C.	C.	C.	++	−	−	−
7.5	C.	C.	C.	C.	C.	++	+	−	−
6.0	C.	C.	C.	C.	C.	++	+	−	−
5.5	C.	C.	C.	C.	C.	++	+	−	−
5.2	C.	C.	C.	C.	C.	C.	+	Tr.	−
5.0	C.	C.	C.	C.	C.	C.	++	+	−
4.6	C.	C.	C.	C.	C.	C.	C.	C.	Tr. +
3.9	C.	C.	C.	C.	C.	C.	C.	C.	C.
3.3	+	+	+	++	++	C.	C.	C.	C.
2.7	+	+	+	+	+	+	+	+	+

Normal Horse Serum.

	pH	2×10^{-3}	1×10^{-3}	5×10^{-4}	2.5×10^{-4}	1.25×10^{-4}	0.6×10^{-4}
Normal horse serum 1.0 cc. + typhoid suspensions 1.0 cc.	5.0	Tr.	Tr.	−	−	−	−
	4.7	+	Tr.	Tr.	−	−	−
	4.4	+	+	Tr.	+	++	++
	3.9	−	Tr.	+	C.	C.	C.
	3.3	−	−	+	+	++	++

pH 3 buffer. The result was instantaneous and intense agglutination, whereas Fig. 3 shows that in the presence of excess serum no agglutination occurs at a pH of 3.

Tables I to IV show the effect of the pH on the amount of normal serum and immune serum to cause agglutination of various organisms.

TABLE II.

Agglutination of Types I and II Pneumococci by Pneumococcus Type I Antiserum at Various C_H. G. P. A. Buffer.

	pH	Dilution of serum. 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Control. No serum.
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type I suspensions 1.0 cc.	8.5	C.	C.	C.	++	Tr.	−	−	−	−	−
	7.1	C.	C.	C.	++	+	−	−	−	−	−
	6.0	C.	C.	C.	++	+	−	−	−	−	−
	5.5	C.	C.	C.	++	+	−	−	−	−	−
	5.2	C.	C.	C.	C.	+	+	+	Tr.	Tr.	−
	5.0	C.	C.	C.	C.	++	+	+	+	Tr.	−
	4.6	++	++	C.	C.	C.	C.	++	+	Tr.	Tr.
	3.9	+	+	+	+	+	++	C.	C.	C.	C.
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type II suspensions 1.0 cc.	8.5	+	+	−	−	−	−	−	−	−	−
	7.1	+	+	Tr.	−	−	−	−	−	−	−
	6.0	++	++	+	Tr.	−	−	−	−	−	−
	5.5	++	++	+	Tr.	−	−	−	−	−	−
	5.2	++	++	+	Tr.	Tr.	−	−	−	−	−
	5.0	++	++	+	+	Tr.	Tr.	−	−	−	−
	4.6	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	−
	3.9	+	+	C.	C.	C.	C.	C.	++	+	+

TABLE III.

Agglutination of Type G Rabbit Septicemia Bacillus by Rabbit >G at Various C_H.

pH	Dilution of serum. 1:10*	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	Control. No serum.
7.9	++	++	+	+	−	−	−	−	−	−	−	−
7.5	++	C.†	++	+	Tr.	−	−	−	−	−	−	−
7.0	++	C.	C.	++	++	+	Tr.	−	−	−	−	−
6.5	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
6.0	C.	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.
5.5	C.	C.	C.	C.	C.	C.	++	++	+	+	+	+
5.0	C.	C.	C.	C.	C.	C.	C.	++	++	++	C.	C.
4.5	+	+	+	C.	C.	C.	C.	C.	C.	C.	C.	C.
2.2	−	−	−	−	−	−	−	−	−	−	−	−

* Represent *serum dilutions*. Divide by 2 to obtain final dilution.

† C = Complete agglutination.

++ = Strong agglutination with sediment but turbid supernatant.

\+ = Slight agglutination.

Tr. = Trace.

TABLE IV.

pH	Dilution of normal serum.										Control. No serum.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
7.0	+	+	−	−	−	−	−	−	−	−	−
6.0	+	+	+	+	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
5.5	+	+	+	+	++	++	++	++	++	++	++
5.0	+	++	++	++	++	C.	C.	C.	C.	C.	C.
4.5	++	++	++	++	++	C.	C.	C.	C.	C.	C.
4.0	+	+	++	++	++	C.	C.	C.	C.	C.	C.
3.0	+	+	+	+	+	++	++	++	++	++	C.
2.0	−	−	−	−	−	−	Tr.	Tr.	Tr.	Tr.	Tr.

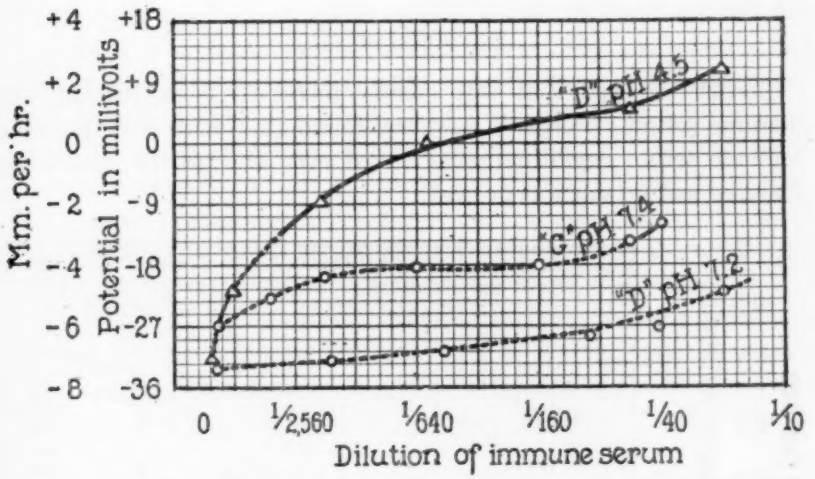

FIG. 4. Effect of immune serum on the potential and agglutination of Type D and Type G at different pH. G. P. A. Buffer.

The results are the same as with egg albumin. In every case the acid agglutination zone is shifted to the alkaline side, and the amount of serum required to agglutinate is a minimum near the isoelectric point of the organism. This result had been noted by Krumwiede and Pratt,[9] and by Michaelis and Davidsohn.[10] It is noticeable also that

[9] Krumwiede, C. K., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1913, xvi, 517.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

the difference between the normal and the immune serum becomes less and less marked as the pH approaches that of the acid agglutination zone of the organism.

Fig. 4 shows the effect of immune serum on the charge and agglutination of Types D and G. As in all the experiments, the agglutination becomes complete as soon as the charge is reduced below 15 millivolts. The figure shows that Type D is difficult to agglutinate because it has a fairly high charge at a pH of 7.2 and the effect of the immune serum is insufficient to reduce this to the critical value. Type G, however, has a lower charge and is much more readily agglutinated. Type D at a pH of 4.5 is easily agglutinated since at this pH the serum has a much greater effect on the charge.

The Effect of Salts.

Bordet[11] showed that salt greatly increased agglutination with immune serum. Porges,[12] however, found that with very powerful immune serum agglutination occurred even though the serum was dialyzed and no salt was present.

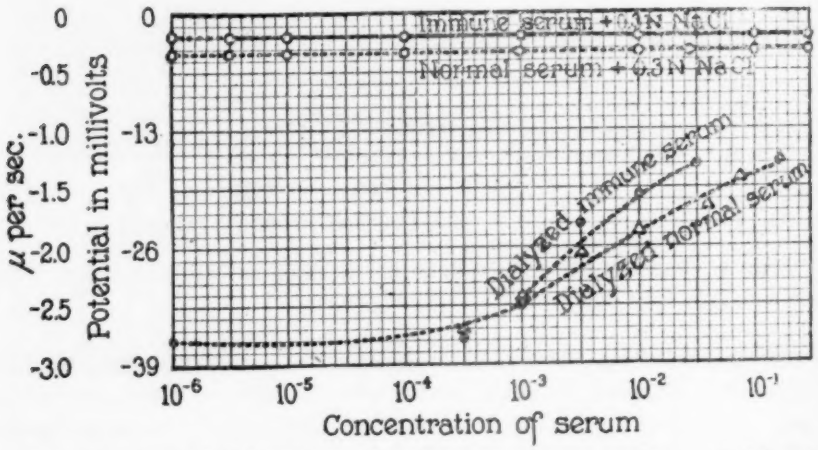

FIG. 5. Effect of dialyzed normal and immune serum on the potential and agglutination of *B. typhosus*. Upper curves show the effect in the presence of 0.3 N NaCl. The potential in these two curves is identical but they have been separated slightly in the figure in order to show the difference in the agglutination.

[11] Bordet, J., Traité de l'immunité dans les maladies infectieuses, Paris, 1920.
[12] Porges, O., *Centr. Bakt., I te Abt., Orig.*, 1905, xl. 133.

The effect of dialyzed normal and of powerful antityphoid horse serum on the potential and agglutination of *Bacillus typhosus* is given in Fig. 5. The two upper curves are the results in 0.3 N NaCl. There was no complete agglutination in the absence of salt and no marked difference between the normal and immune serum, although both affect the potential. (The serum was prepared by dialysis against distilled water and then dissolved by the addition of a small amount of NaOH. Conductivity measurements showed that the total concentration of salt was less than 0.001 N; *i.e.*, too small to cause the noted effect on the potential.) In the presence of salt, on the other hand, there is no effect on the potential but agglutination occurs in very high dilution with the immune serum and to a much less extent with the normal serum. This experiment shows that the effect of the serum on a suspension of bacteria in *concentrated salt* solution is not primarily on the charge but on the cohesive force. The serum raises the cohesive force and hence the critical charge to a value greater than the potential carried by the organism and they therefore agglutinate. The effect of the serum on the cohesion is shown in Fig. 6. The upper part of the figure shows that the addition of serum raises the cohesion to the value in distilled water; *i.e.*, it prevents the salt from decreasing the attractive force and thereby lowering the critical potential. The lower part of the figure shows the converse experiment; *i.e.*, the effect of salt on a film of washed, and of sensitized organisms. The salt decreases the cohesion of the washed organisms very markedly but has no effect on the cohesion of the film sensitized with serum.

The effect of varying both the salt and the serum concentration on the agglutination is shown in Table V. As the serum concentration is increased, the salt concentration in which complete agglutination occurs widens on both sides from 0.10 N. The lower limiting concentration of salt remains at about 0.01 N, however, and does not continue decreasing as the serum increases. In other words, the effect is not additive, but there is a critical concentration of serum beyond which there is little or no effect on the concentration of salt needed to agglutinate. This "critical" salt concentration corresponds to the point at which the charge on the organisms is about 10 millivolts; *i.e.*, just under the critical potential. This is the result expected if the

agglutinin forms a film on the surface of the organism. As soon as the layer is complete the addition of excess serum will have no effect. If this assumption is correct, it follows from Table V that agglutination occurs when the surface is about one-eighth covered. The inhibiting effect of strong salt solution is, as usual, due to the decrease in the cohesive force, when the organisms are not completely covered with immune body.

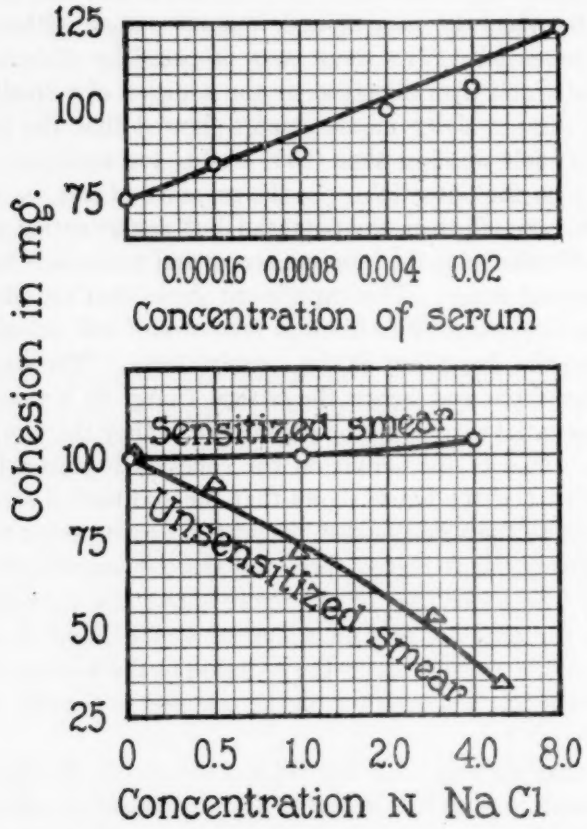

FIG. 6. Upper half, effect of the concentration of immune serum on the cohesive force between two films of *B. typhosus* in 0.10 N NaCl. Lower half, effect of concentration of NaCl on the cohesive force between, a, smears treated with immune serum and, b, untreated smears.

It is evident from the foregoing that the agglutination may be considered as caused by the salt, as Bordet stated. The serum, however, does not *sensitize* the bacteria but *protects* it from the salt so that the latter does not reduce the cohesive force. If we study the effect of salts and acids on the agglutination and charge of organisms sensitized with immune serum, we should expect then to obtain curves similar

TABLE V.

Influence of NaCl Concentration on Agglutination with Dialyzed Normal and Immune Serum.

Concentration of immune serum	Agglutination after 24 hrs. at 20°C.								
	Concentration of NaCl.								
	0	0.001	0.003	0.01	0.03	0.1	0.3	1.0	1.4
1:150	+	+	+++	C.	C.	C.	C.	C.	C.
1:300	−	−	+++	C.	C.	C.	C.	C.	C.
1:600	−	−	+++	C.	C.	C.	C.	C.	C.
1:1,200	−	−	++	C.	C.	C.	C.	C.	C.
1:2,400	−	−	+	C.	C.	C.	C.	C.	++
1:4,800	−	−	−	++	C.	C.	C.	C.	+
1:9,600	−	−	−	+	++	C.	C.	++	−
1:19,200	−	−	−	−	+	C.	++	+	−
0	−	−	−	−	−	+	+	−	−
Concentration of normal serum.									
1:12	+	++	+++	C.	C.	C.	C.	C.	C.
1:24	−	−	+	C.	C.	C.	C.	+++	+++
1:48	−	−	−	+++	+++	+++	++	+	+
1:96	−	−	−	++	+++	++	+	+	−
1:192	−	−	−	−	+	++	+	−	−
μ per sec.	−2.5	−1.3	−1.0	−0.7	−0.4	−0.15	0	0	0
Millivolt potential.	−32	−17	−13	−9.0	−5.6	−0.19	0	0	0

to those given in the preceding paper with the exception that the stable zone in high concentrations of salt would not appear and the agglutination should be found to depend entirely on the potential. A summary of a number of such experiments is given in Fig. 7. The serum concentration was 1:500 in all cases; *i.e.*, in excess. The figure shows that with the exception of strong acid solution, complete agglutination occurred whenever the potential was reduced below 15

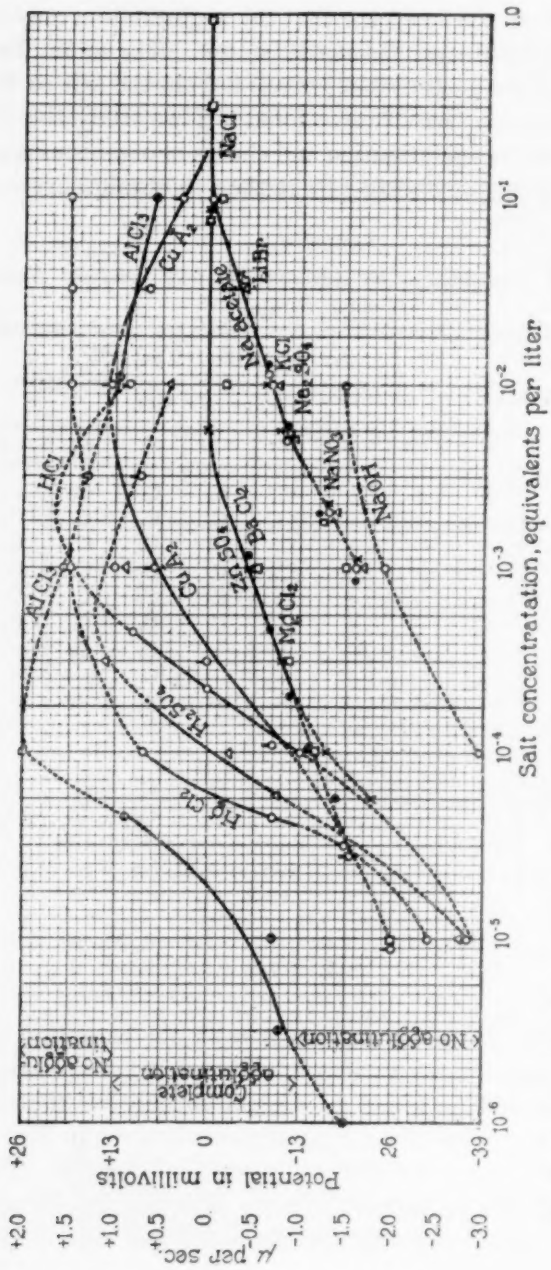

FIG. 7. Effect of the salt concentration on the agglutination and potential of *B. typhosus*, sensitized with immune serum.

millivolts, and there is no stable zone in concentrated salt. The effect of the strong acid is due partly to destruction of the antibody and partly to the fact that the combination of the antibody with the bacteria is less complete in acid solutions.[13]

The results also show that the effect of all monovalent cations (except hydrogen) was identical both as regards potential and agglutination. The valency and nature of the anion have no effect. This is the usual result when the particles are negative. The bivalent cations agglutinate in much lower concentration. The trivalent curves are not comparable owing to changes in the pH.

SUMMARY.

1. The addition of proteins or serum to suspensions of bacteria, (*Bacillus typhosus* or rabbit septicemia) at different pH widens the acid agglutination zone and shifts the isoelectric point to that of the added substance.

2. The amount of serum required to agglutinate is much less near the acid agglutination point of the organisms.

3. The addition of immune serum prevents the salt from decreasing the cohesive force between the organisms, and agglutination therefore is determined solely by the potential, provided excess immune body is present. Whenever the potential is decreased below 15 millivolts the suspension agglutinates.

[13] This point is taken up in the following paper.

THE FLOCCULATION OF BACTERIA BY PROTEINS.

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The effect of the addition of proteins on the stability of suspensoid colloids has been the subject of numerous investigations. Ordinarily the proteins act to increase the stability of the suspension, hence their use as protective colloids. Under certain circumstances, however, they have been found to produce flocculation instead of protection. Neisser and Friedemann (1904) found that NaCl in a dilution incapable of producing flocculation by itself can coagulate mastic sol if one part of gelatin per million is present; blood serum, leech extract, and bacterial extract behaved in the same manner. Walpole (1913) reported that gelatin, albumin, and globulin, when added in high dilutions to oil emulsions or gold or mastic sols increased their sensitiveness to flocculation both by acids and salts. Brossa and Freundlich (1914) showed that dialyzed serum albumin, when added to $Fe(OH)_3$ sol, diminished the positive charge on the colloidal Fe $(OH)_3$ and caused it to be flocculated by concentrations of salt that were without effect upon the pure sol.

In the present investigation, the effect of pure proteins on the stability of bacterial suspensions at different H ion concentrations was studied. Several species of bacteria were used, but the most instructive results were obtained with a strain of *Bacterium coli*. This is because the point of optimum flocculation (the isoelectric point) for this organism lies in a very acid range, which leaves a wide interval between it and the isoelectric points of the different proteins studied. According to Beniasch (1912), *Bacterium coli* is not agglutinable by H ions. We have not found any recently isolated strains that would agglutinate in any of the buffer mixtures used by Beniasch; but most, though not all, strains of this organism will agglutinate in dilute acids in the absence of salt, at reactions ranging from pH 1.6 to 3.0. Putter

(1921) has also found *Bacterium coli* to agglutinate with acids in the absence of salt. The strain with which most of our work was done underwent a curious mutation about 2 months after it was first isolated. Our records show that on December 11 no agglutination took place in any of the acetate or lactate buffer mixtures; 3 days later the organism, with all its other characteristics unchanged, was found to agglutinate at pH 3.2 (negative at 3.0 and 3.5), both in the acetate and lactate buffer mixtures, but not in the phthalate buffers of Clark and Lubs (1917). No further change has been observed to the present time.

In all of these experiments, *Bacterium coli* was grown on beef extract peptone agar plates. The growth was suspended in 0.85 per cent NaCl, filtered through paper, centrifugated, then centrifugated three times out of distilled water. In most of the experiments, a temperature of 40°C. was employed. Flocculation was observed macroscopically; no test was considered positive unless definite macroscopic flocks were formed, which settled out to leave a clear supernatant fluid.

Lactic Acid-Sodium Lactate Buffer Mixture.

0.1 N lactic acid, *cc.*	0.6	1.2	2.4	5.0	10.0				
N lactic acid, *cc.*						2.0	4.0	8.0	16.0
Water, *cc.*	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium lactate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Acetic Acid-Sodium Acetate Buffer Mixtures.

0.1 N acetic acid, *cc.*	0.6	1.2	2.4	5.0	10.0				
N acetic acid, *cc.*						2.0	4.0	8.0	16.0
Water, *cc.*	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium acetate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

Phosphate Buffer Mixtures.

0.1 N potassium dihydrogen phosphate, *cc.*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
0.1 N sodium hydroxide, *cc.*	0.37	0.57	0.86	1.26	1.78	2.36	2.96	3.50
Water, *cc.*	14.63	14.43	14.14	13.74	13.22	12.64	12.04	11.50
pH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2

Lactic acid-lactate and acetic acid-acetate buffer mixtures were made up according to the method of Beniasch (1912), which gives mixtures of uniform salt concentration (0.025 M). This uniformity of salt concentration is essential, as the salt effect is very high in some cases. The phosphate buffer mixtures were made up according to the directions of Clark and Lubs (1917), except that they are made twice as dilute, to make the salt concentration equal to that of the Beniasch buffer mixtures.

Flocculation of Bacteria with Gelatin.

Isoelectric gelatin was prepared by the method of Loeb (1919). Suspensions of *Bacterium coli* were incubated with varying dilutions of gelatin, both in buffer mixtures (Table I) and in the absence of salt (Table II).

TABLE I.

Bacterium coli Suspension with Gelatin.

1.0 cc. buffer mixture + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	Acetate buffers.								Lactate buffers.		
0	−	−	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	+	+	−	−	−
1:40,000	−	−	−	−	×	×	×	×	+	−	−
1:4,000	−	+	×	×	×	+	−	−	−	−	−
1:400	−	−	+	−	−	−	−	−	−	−	−
pH	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

In Table I it is to be noticed that with the highest concentration of gelatin, agglutination occurs only at pH 4.7, the isoelectric point of the gelatin. In the 1:4,000 dilution, the zone of flocculation widens, especially on the acid side, but it also includes the pH 5.0 tube. Beginning with the 1:40,000 dilution of gelatin, the zone of flocculation shifts over to the acid side, even becoming more acid than the flocculation zone of the control. It will be noted that there is an absence of flocculation with the higher concentrations of gelatin in the more acid tubes, forming a so called "proagglutinoid zone."

TABLE II.

Bacterium coli Suspension with Gelatin.

Acid and water to make 1.0 cc. + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	0.002 N HCl.							0.02 N HCl.			0.2 N HCl.		
	0	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.
0	–	–	–	–	–	–	×(3.0)	×	×	×	×(1.6)	–	–
1:4,000,000	–	–	–	–	–	–	×	×	×	×	×	–	–
1:400,000	–	–	–	–	–	×(3.4)	×	×	×	×	×	–	–
1:40,000	–	–	–	–	–	×	×	×	×	×	×	×(1.3)	–
1:4,000	–	–	–(5.2)	×(4.6)	×(4.0)	–	–	–	–	–	–	–	–
1:400	–	–	×(5.0)	×(4.6)	–(4.2)	–	–	–	–	–	–	–	–

Temperature = 40°C. Flocculation complete in 30 minutes. Reactions were determined colorimetrically as soon as flocculation was evident; the figures in parentheses indicate the pH.

Cataphoresis experiments with a micro apparatus similar to that of Putter (1921) show that the bacteria in this zone carry a positive charge.

In Table II, the experiment is repeated in the absence of salt. The general results are similar to those found in Table I, except that the zone of flocculation of untreated *Bacterium coli* is found to lie between pH 1.6 and 3.0. It will be noted that when isoelectric gelatin is added to a suspension of *Bacterium coli*, the mixture is more alkaline than pH 4.7; this is because the bacterial suspension, though carefully washed and in pure water, maintains a pH of 6.8 to 7.0 even though unprotected from the CO_2 of the air.

The use of indicators in determining the H ion concentration of unbuffered or poorly buffered solutions calls for a word of explanation. Tizard (1910) has shown with methyl orange and methyl red that serious errors can be made. In a series of unpublished experiments made in collaboration with C. B. Coulter, in which all results were checked electrometrically, it was found that a carefully purified methyl red indicator could be used without serious error in ranges from pH 4.6 to 5.4. With the various sulfonephthalein indicators, which can be made up in aqueous solution, it was found that if the dye be adjusted so that its pH is not far from the pH of the solution tested, quite reliable results could be obtained. Thus, brom thymol blue indicator, adjusted so that when viewed in thin layers it matched the pH 6.8 standard, can be used over the range pH 6.4 to 7.2, without an error greater than 0.2 pH. Without adjustment of the indicator for particular ranges, the error may be much greater than this.

The results obtained in these experiments are strikingly similar to those found by Michaelis and Davidsohn (1912) in the precipitation of nucleic acid (isoelectric at about pH 0.7) by serum albumin, which is isoelectric at pH 4.8. Maintaining a constant concentration of nucleic acid, these authors found a concentration of albumin that produced a precipitate whose optimum occurred at pH 4.1 to 4.4. On lowering the concentration of albumin, the optimum now shifted to the acid side, and the optimal zone was greatly broadened. This is essentially like what we have described in Tables I and II. Michaelis and Davidsohn explained their results on purely electrical grounds. Nucleic acid is negatively charged at all reactions alkaline to pH

0.7; albumin is positively charged at all reactions acid to pH 4.8. At all intermediate reactions, the two colloids are oppositely charged; they tend to attract each other and neutralize their charges; when this occurs, their combination is isoelectric and flocks out. Moreover, at the acid end of this intermediate zone, the nucleic acid particle is weakly, whereas the albumin is strongly charged, hence a smaller amount of albumin will be needed to combine with and discharge the nucleic acid than at the other end of the zone, where the strongly charged nucleic acid would require larger amounts of the more weakly charged albumin to neutralize it. This view is capable of application to such phenomena as the flocculation of *Bacterium coli* by gelatin at pH 5.0 and 3.0 (Table I), points that lie outside of the intermediate zone. As these authors point out in another paper (1913), any solution of ampholyte at every pH exists in three forms,—as anion, as cation, and as undissociated molecule; it is the relative concentration of these three species that changes with H ion concentration. Cationic gelatin exists, therefore, at pH 5.0; anionic gelatin exists at pH 3.0. At pH 5.0, one might conceive that the bacterial cell (which is here negatively charged) would unite with the small amount of gelatin cations present; the gelatin cations being thus removed from solution, more would be liberated, this process continuing until a state of equilibrium between gelatin cations in solution and gelatin cations combined was reached. If the charge on the cell-gelatin complex is below a certain threshhold value when equilibrium is reached, agglutination may occur, otherwise not. As it is the pH that determines the amount of gelatin cations in solution, it likewise determines the amount that can be combined when equilibrium is reached; hence it can be understood why agglutination, though occurring at pH 5.0, might not occur at pH 5.3. The same explanation would apply to the flocculation of *Bacterium coli* by proteins at reactions more acid than the flocculation zone of the untreated bacteria.

It is more difficult to apply this theory to the pro-zone observed in these experiments. Thus (Table I) when the gelatin concentration is 1:400, no agglutination of the bacteria occurs at reactions from pH 4.4 to 3.0, though more dilute gelatin causes flocculation. The bacteria in this zone are charged positively. If a primary electrical neutralization occurred, it must have been followed by further com-

bination with gelatin cations, and this must have required a different mechanism. We cannot exclude the possibility that the primary cause of union between bacteria and protein is non-electrical (perhaps a surface tension phenomenon), and that electrical neutralization is a secondary effect which may or may not occur.

Flocculation with Crystallized Egg Albumin.

Crystallized egg albumin was obtained by the method of Hopkins and Pinkus (1898); this was dialyzed to remove the ammonium sulfate. Experiments were conducted in buffer mixtures only (Table III).

TABLE III.

Bacterium coli Suspension with Egg Albumin.

1.0 cc. buffer mixture + 0.5 cc. albumin solution + 0.5 cc. *coli* suspension.

Concentration of albumin.	Acetate buffer.	Lactate buffers.								
0	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	+	−	−	−
1:40,000	−	−	−	−	+	+	+	+	−	−
1:4,000	−	−	×	×	×	×	×	+	−	−
1:400	−	−	×	×	−	−	−	−	−	−
pH	5.0	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 1 hour. + = agglutination within 4 hours.

Agglutination of this strain of *Bacterium coli* did not occur at pH 4.8, the isoelectric point of the albumin. A second strain of this organism was agglutinated at pH 4.7 by an albumin concentration of 1:150.

Flocculation with Protalbumose and Heteroalbumose.

These albumoses were prepared from Witte's peptone by the method of Pick (1898). The heteroalbumoses gave a clear solution when boiled and adjusted to pH 7.0; the protalbumose required no adjustment to give a clear solution.

The protalbumose was completely soluble at all reactions tested. The heteroalbumose, in a concentration of 1:400, was turbid at reac-

tions between pH 4.4 and 6.4, with a maximum turbidity at pH 5.3 and 5.6.

TABLE IV.

Bacterium coli Suspension with Protalbumose.

1.0 cc. buffer mixture + 0.5 cc. protalbumose solution + 0.5 cc. *coli* suspension.

Concentration of protalbumose.	Lactate buffers.								
0	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	×	×	−	−	−
1:400,000	−	−	−	−	×	×	×	×	−
1:40,000	−	−	−	−	×	×	×	×	−
1:4,000	−	−	−	×	×	×	×	−	−
1:400	−	×	×	×	×	×	×	−	−
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

Temperature = 40°C. All agglutinations were complete in 1 hour.

TABLE V.

Bacterium coli Suspension with Heteroalbumose.

1.0 cc. buffer mixture + 0.5 cc. heteroalbumose solution + 0.5 cc. *coli* suspension.

Concentration of heteroalbumose.	Phosphate buffers.			Acetate buffers.									Lactate buffers.		
0	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
1:4,000,000	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
1:400,000	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−
1:40,000	−	−	−	−	−	−	−	−	+	×	×	×	×	×	−
1:4,000	−	−	−	−	−	×	×	×	×	×	×	×	−	−	−
1:400	−	+	×	×	×	×	×	×	×	×	+	−	−	−	−
pH	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 3 hours.

Flocculation with Edestin.

Crystalline edestin was prepared from hemp seed by the method of Osborne (1901). This substance was found to be practically insoluble in water or in the buffer mixtures used at reactions between pH 5.6 to 9.6. Rona and Michaelis (1910) report pH 6.9 as the

isoelectric point of edestin, though in a later paper Michaelis and Mendelssohn (1914) give it a value of pH 5.6.

In the experiment shown in Table VI, a 0.25 per cent suspension of edestin in distilled water was dissolved by the addition of minimal alkali; the solution was immediately distributed in the buffer mixtures and incubated at 40°C. for 30 minutes. The 1:1,600 dilution of edestin gave a small precipitate in the pH 5.3 tube, and a heavier flocculent precipitate in the tubes alkaline to this. *Bacterium coli* suspension was then added without stirring up the precipitate that had formed.

TABLE VI.

Bacterium coli Suspension with Edestin.

1.0 cc. buffer mixture + 0.5 cc. edestin solution + 0.5 cc. *coli* suspension.

Concentration of edestin.	Phosphate buffers.		Acetate buffers.								
0	−	−	−	−	−	−	−	−	−	−	+
1:1,600,000	−	−	−	−	−	−	−	−	−	−	+
1:160,000	−	−	−	−	−	−	−	−	+	×	×
1:16,000	−	−	−	×	×	×	×	×	×	+	−
1:1,600	−	+	×	−	−	−	−	−	−	−	−
pH	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

Flocculation with Hemoglobin.

Crystallized oxyhemoglobin was prepared from the blood of the horse, the dog, and the guinea pig, some by the method of Hoppe-Seyler (1903), and some by the method of Dudley and Evans (1921). No marked differences in behavior were observed between these crystallized oxyhemoglobins and other solutions prepared by laking washed erythrocytes and centrifugating out the stroma. In the results obtained, there were some differences between the hemoglobins of different species of animals, and between the oxy- and methemoglobins of the same species.

It will be observed that in the most concentrated hemoglobin that we used (Table VII) the zone of flocculation extended only as far toward the alkaline side as pH 6.2 (in some experiments,

to pH 6.4). As we have observed that when we added a 1 per cent pure oxyhemoglobin solution to two or three volumes of *Bacterium coli* suspension, flocculation occurred without any adjustment of reaction, we suspected that salts interfered with the agglutination near the isoelectric point of the hemoglobin. In one experiment, where H ion concentrations were determined electrometrically, flocculation of the bacteria occurred in the absence of salt at pH 6.71 and points acid to this, the concentration of hemoglobin being 1:400.

It is also to be noted in Table VII that there is no pro-zone with the higher concentrations of hemoglobin, such as we have uniformly

TABLE VII.

Bacterium coli Suspension with Guinea Pig Oxyhemoglobin.

1.0 cc. buffer mixture + 0.5 cc. oxyhemoglobin solution + 0.5 cc. *coli* suspension.

Concentration of hemoglobin.	Phosphate buffers.				Acetate buffers.				
0	−	−	−	−	−	−	−	−	−
1:250,000	−	−	−	−	−	−	−	−	−
1:50,000	−	−	−	−	−	−	×	×	×
1:10,000	−	−	−	−	×	×	×	×	×
1:20,000	−	+	×	×	×	×	×	×	×
1:400	−	+	×	×	×	×	×	×	×
pH	6.4	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4

Temperature = 20°C. × = agglutination within 1 hour. + = agglutination within 12 hours.

found with the other proteins studied. We did not extend the series more acid than pH 4.4, because the greenish brown color of the solutions showed that we no longer had oxyhemoglobin. When *Diplococcus pneumoniæ* suspensions were used instead of *Bacterium coli*, a pro-zone was obtained; when the hemoglobin concentration was 1:400, the bacteria were charged positively at pH 5.0 and reactions acid to this, and remained non-agglutinated. When, instead of bacteria, we used an aqueous suspension of cellulose nitrate as substrate, hemoglobin in dilutions of 1:2,000 to 1:10,000 caused agglutination of the cellulose nitrate at reactions between pH 6.2 to 7.0 (in the absence of salt); with higher dilutions of hemoglobin, flocculation took place at more acid reactions.

The experiments described above establish the influence of proteins upon the stability of bacterial suspensions at different H ion concentrations, and relate this influence to the isoelectric point of the added protein and its concentration. It must be noted that bacteria are not the only suspensions that are effected by proteins in this way. We have tested the effect of proteins on sols of cellulose nitrate, cellulose acetate, and paraffin emulsions, with results that closely parallel our experiments with bacteria. Although Walpole (1913) does not relate his results to the isoelectric point of the protein, it seems clear, from the curve given by him, that gelatin, albumin, and globulin, affect the stability of oil emulsions and mastic and gold sols at different concentrations of H ions in the same manner that these substances affect bacterial suspensions. The same may be said of the action of albumin on $Fe(OH)_3$ sol at different salt concentrations, as reported by Brossa and Freundlich (1914). Of the same import are the observations of Putter (1921) that in the presence of peptone, acids change the sign of the charge on *Bacterium coli*, and of Coulter (1922) who shows that erythrocytes in contact with specific sensitizer, or even with normal homologous or heterologous sera, agglutinate most promptly when the pH is such that the euglobulins of these sera are isoelectric.

Loeb (1920) has shown that when collodion membranes are treated with proteins, a combination takes place between the collodion and the protein, and the membrane becomes isoelectric near the H ion concentration at which the protein is isoelectric. Reactions acid to this point now charge the membrane positively. It is obvious that the effect of proteins upon bacteria (and probably upon other suspensions and emulsions) is of the same nature. Where concentrations of about 0.25 per cent of protein are used, the bacteria agglutinate at or near the isoelectric point of the protein; increasing the H ion concentration beyond this point causes the original charge upon the bacteria to be reversed, and prevents agglutination.

SUMMARY.

1. The effect of adding pure proteins to bacterial suspensions at different H ion concentrations has been studied.

2. The zone of flocculation of protein-treated bacteria bears a significant relationship to the isoelectric point of the protein used. With the higher concentration of protein, agglutination occurs at or near the isoelectric point of that protein; at reactions acid to this, the bacteria carry a positive charge and are not agglutinated. With diminishing concentration of protein, the zone of flocculation shifts toward and goes beyond that characteristic of the untreated bacteria. This occurs both in the presence and absence of salts.

3. A diversity of other suspensions, such as sols of gold, mastic, cellulose nitrate, cellulose acetate, $Fe(OH)_3$, oil emulsions, and erythrocytes, have been found by ourselves and others to exhibit a similar altered stability when treated with proteins in the same way.

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FURTHER STUDIES ON EOSIN HEMOLYSIS.

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It is a well known fact that if a dilute solution of a photosensitive substance such as eosin is added to a suspension of washed red blood cells and the mixture is exposed to sunlight, hemolysis of the red cells promptly takes place, while no action is observed when the mixture is kept in the dark. Busck (1) and later Sellards (2) found that the addition of certain substances such as blood serum and egg white to solutions of photobiologic sensitizers results in decreasing or completely inhibiting the toxic action, but no protection is afforded to cells by the addition of glucose, starch, or gelatin. Recognizing that there are fundamental differences in the chemical make-up of those substances which afford protection and those which do not, Schmidt and Norman (3) carried out experiments to determine the relation between the amino-acid content of the protein molecule and protective action. They found that eosin hemolysis can be prevented by the addition of tyrosine, tryptophane, and proteins which contain these amino-acids. Certain other organic compounds which contain the hydroxyphenyl ring also afford protection. They pointed out that the inability of gelatin to protect red blood cells against eosin hemolysis is due to the absence of the above essential amino-acids. As a tentative explanation of this phenomenon, they consider that the protection afforded by certain substances against the photodynamic effect of eosin may possibly be due to the absorption of the active rays by the protective substance.

Since the publication of these experiments, we noted a striking similarity between the substances which protect red blood cells against hemolysis by eosin, and the substances which were found by Gortner and Holm (4) to react with the Folin and Denis (5) phosphotungstic-phosphomolybdic reagent to give a characteristic blue color. These

substances were found by Gortner and Holm to include tyrosine, tryptophane, uric acid, α-methyl indole, and ferrous iron. Abderhalden (6) states that the list also includes oxyproline and oxytryptophane. The property which is possessed in common by all of these substances is that they are easily oxidizable.

Experimental work was accordingly undertaken to determine whether inorganic reducing substances in addition to tyrosine, tryptophane, and proteins which contain these amino-acids in the molecule can afford protection to red cells against eosin hemolysis, and our results appear to answer this question in the affirmative. As in the previous work, 0.5 cc. of a 5 per cent saline suspension of red blood cells (ox or sheep) was placed in each of a number of small test-tubes, and to each, 1 cc. of a 1:10,000 dilution of eosin (Grübler's) in salt solution was added. The substances to be tested for protective action were likewise made up in normal saline solution in the concentrations, as given in Table I, and the reaction was adjusted to approximate neutrality. The tubes were placed in direct sunlight for 30 minutes and after exposure they were immediately placed in the ice chest. The tubes were inspected at the end of several hours to determine the amount of lysis which had taken place. Control tubes which were kept in the dark eliminated factors other than that of photodynamic action. The experimental results are given in Table I. They indicate that inorganic reducing substances afford marked protective action to red blood cells against eosin hemolysis. The list of inorganic reducing substances which may be used in experiments of this type is limited, since many of the best reducing agents such as ferrous chloride, ferrous sulfate, and ferrous ammonium sulfate, yield solutions of high acidity, and when these are added to red cells the latter are agglutinated. Oxyproline and oxytryptophane were not available for experimental work. It is doubtful whether the former substance can protect red cells against the toxic action of eosin, since gelatin, which contains 14 per cent (7) of this substance, lacks protective ability. Marked protection was shown by each of the two preparations of histidine. Both gave a trace of blue color when tested by the Folin and Denis reagent, indicating the possible presence of tyrosine. Valine, serine, proline, creatinine, and cinnamic acid afford no protection, while marked protection is afforded by skatole and tryptophane.

Since the action of the protective substance appears to be that of a reducing agent, it seemingly follows that eosin hemolysis is a phenomenon involving oxidation. Hemolysis may be wholly prevented by placing the eosin-red cell mixture in a highly evacuated glass tube or by saturating the cells with illuminating gas or hydrogen. These observations are in agreement with the statement of Sellards, that an atmosphere of hydrogen is as effective as total darkness in preventing

TABLE I.

The Effect of the Addition of Certain Substances on the Hemolysis of Red Cells by Eosin.

Substance added.	Concentration.*	Result.
Sodium chloride (control)		Complete hemolysis.
Valine	M/10	" "
Serine	M/10	" "
Proline	M/10	" "
Cinnamic acid	M/10	" "
Creatinine	M/30	" "
Tryptophane	M/30	No hemolysis.
Skatole	Saturated solution.	" "
Sodium sulfite	M/30, M/90	" "
Sodium thiosulfate	M/30	" "
Ferrous lactate	M/30	" "
Potassium ferrocyanide	M/30	" " Some hemolysis over night.
Histidine 1	M/20	No hemolysis.
Histidine 2	M/20	" "

* Sufficient NaCl was added to each of these solutions to make them isotonic.

the toxic action of eosin in sunlight. It must be admitted that in certain instances the reducing agent may react with the fluorescent substance and in this way partially inhibit its toxic action. Thus, when sodium sulfite is added to a solution of eosin and the mixture is exposed to sunlight, the latter substance is rapidly reduced to fluorescein.

The action of sunlight in accelerating oxidative reactions is a well known phenomenon. Bilirubin when exposed to sunlight is oxidized to biliverdin (8), many of the vegetable oils are oxidized by light, and the bleaching of the triphenylmethane dyes (9) is a phenomenon in

which the dye itself appears to catalyze the oxidation which is accelerated by the sun's rays. We have noted that the bleaching of eosin solutions when exposed to sunlight may be markedly inhibited by the addition of tryptophane, while alanine, glycocoll, and phenylalanine afford little or no protection.

Three possible reactions may conceivably take place in the oxidation of the protective protein or amino-acid. The first is oxidative deamination which yields an α-ketonic acid. For tyrosine the reaction is represented by the equation:

$$C_6H_4OH \cdot CH_2 \cdot CHNH_2 \cdot COOH + O = C_6H_4OH \cdot CH_2 \cdot CO \cdot COOH + NH_3$$

This is a universal reaction and represents a step in the normal catabolism of the amino-acids. The protective action against eosin hemolysis afforded by tyrosine and tryptophane cannot be due to this reaction, since it is not at all specific for these two amino-acids. Moreover, ammonia, which itself possesses hemolytic properties, is set free in the reaction. It is possible that the latter substance may be a factor which is concerned in eosin hemolysis. Experimental work, however, does not appear to support this hypothesis. A series of test-tubes containing 100 mg. each of glycocoll, alanine, and tryptophane, dissolved in 5 cc. of normal salt solution and 1 cc. of 1:10,000 eosin was exposed to sunlight for 1 hour. At the end of this time the eosin was decolorized in the tubes containing glycocoll and alanine. The ammonia was taken up with permutit and subsequently set free by the addition of NaOH and the solutions were Nesslerized. A trace of ammonia was found in each instance while none was evident in the control tubes which had been kept in the dark. After 4 hours exposure, the tryptophane solution gave an ammonia content which corresponds to approximately 0.1 cc. of a 0.03 normal solution, about half of the amount necessary to hemolyze completely the dosage of red cells. A series of test-tubes, each containing 1 cc. of a 0.1 normal solution respectively of glycocoll, alanine, phenylalanine and tryptophane, and 1 cc. of 1:10,000 eosin solution was exposed to sunlight for 1 hour, and after exposure 0.5 cc. of a 5 per cent saline suspension of washed sheep cells was added to each tube, the mixtures were shaken and placed in the ice chest. Only a trace of hemolysis was shown by the tubes after standing over night. In this connection it might be

mentioned that the ammonia, which is formed as a result of the oxidation of the amino-acid, is probably not free but is combined with the ketonic acid.

Neuberg (10) studied the mechanism of the reaction which takes place when solutions of certain amino-acids to which uranium salts have been added are exposed to sunlight. In addition to oxidative deamination, he found that CO_2 was split off from the carboxyl group yielding an aldehyde, the reaction being similar to that which takes place when an amino-acid is oxidized with H_2O_2. When serine is exposed to sunlight or is oxidized with H_2O_2, glycol aldehyde is formed according to the reaction:

$$\begin{array}{l} \text{COOH} \\ | \\ \text{CHNH}_2 \\ | \\ \text{CH}_2\text{OH} \end{array} + \text{O} = \begin{array}{l} \text{CHO} \\ | \\ \text{CH}_2\text{OH} \end{array} + \text{CO}_2 + \text{NH}_3$$

This reaction like the one discussed previously is universal, and fails to explain the specific reducing action of tyrosine and tryptophane.

It is a well recognized fact that benzene and a large number of aromatic substances of varied types undergo substitution of the hydrogen atoms in the nucleus to a more or less marked extent, and *in vitro* this reaction can be brought about by ozone, H_2O_2, and by photochemical action (11). We have attempted to test the possible application of this reaction to the subject of eosin hemolysis. Suspensions of washed red cells were treated respectively with H_2O_2 (saline solution of the neutralized product), H_2O_2 plus a small amount of catalase, and H_2O_2 plus platinum black. In each instance the oxyhemoglobin was converted into methemoglobin, but hemolysis did not take place. On saturating red cells with ozone a similar result was obtained. Tubes containing red cells to which platinum black and colloidal palladium were added, showed no hemolysis after exposure for a half hour to sunlight. Evidently hemolysis cannot be brought about with the aid of H_2O_2. The fact that substances which contain either the hydroxyphenyl ring which apparently facilitates the introduction into the nucleus of other OH groups, or the indole ring which is easily oxidized to indoxyl and then to indigo blue (Abderhalden (12) has noted that tryptophane and adrenalin are sensitive to light), can afford protection

to red blood cells against the toxic action of eosin, leads us to believe that we are dealing with a special type of oxidation which is markedly accelerated by fluorescent substances. The highly specific action of tyrosine and tryptophane as reducing agents likewise indicates that these amino-acids which are contained in the protein molecule are attacked and undergo oxidation as a result of the photodynamic action of eosin. It is not possible at this time to state how far this oxidation proceeds or the mechansim whereby lysis takes place. It appears logical to assume that the oxidation concerns itself with the proteins of the stroma and this results in the necrosis of the cell (13).

Bovie (14) has demonstrated that coagulation of proteins can be brought about by exposure to ultra-violet light. This reaction is presumably one of denaturation (15) and does not involve oxidation. To eliminate the possibility of denaturation being a factor in eosin hemolysis, the following experiment was carried out: a test-tube containing 2 cc. of horse serum and 0.1 cc. of 1:1,000 eosin was exposed to sunlight for a period of 4 hours and subsequently incubated at 37°C. There was no visible coagulation.

SUMMARY.

Additional experimental work on the subject of eosin hemolysis has been carried out. This indicates that red cells may be protected against the toxic action of eosin in sunlight by the presence of inorganic reducing agents. It is pointed out that a marked parallelism exists between the substances which react with the Folin and Denis reagent and the compounds which afford protection to red cells against the photodynamic action of eosin. The property which is possessed in common by all of the substances is that they are easily oxidized, and their ability to protect red cells lies in their power of reduction. The toxic action of eosin probably involves the oxidation of tyrosine and tryptophane which are contained in the protein molecules of the stroma.

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THE CALIBRATION OF THE OSTERHOUT RESPIRATORY APPARATUS FOR ABSOLUTE QUANTITIES OF CARBON DIOXIDE.

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In the course of a piece of work on the amount of carbon dioxide produced by sea anemones it was found desirable to use the respiratory apparatus devised by Osterhout (1918), and as it was necessary in this work to determine the absolute quantity of carbon dioxide eliminated by these animals, an attempt was made to calibrate this apparatus for such determinations. The method finally adopted seems to be of enough general importance to justify a brief statement of it.

The Osterhout apparatus consists of a closed system in which the air is made to circulate by means of a pump. The air passes from a chamber, in which the organism is confined and in which consequently the carbon dioxide is produced, either directly to a glass tube containing an indicator in solution or indirectly to this tube through a U-tube filled with fragments of sodium hydroxide. In the direct course the air containing the carbon dioxide is forced to bubble through the indicator solution, thus causing it to change its tint. In the indirect course the air is relieved of its carbon dioxide by contact with the sodium hydroxide, and, after purification, it is bubbled through the indicator thus washing out the contained carbon dioxide and bringing the indicator back to its original color. From the tube containing the indicator the air returns by a direct course through the pump to the chamber in which the organism is confined. A figure of the apparatus is given by Osterhout (1918). In taking readings with this apparatus the time in seconds required for the indicator to change from one tint to another as compared with standard solutions of known pH value is recorded and the rate

of this change is assumed to be identical with that of the excretion of carbon dioxide by the organism.

In calibrating the apparatus various means were used to produce a constant and measurable flow of carbon dioxide as a substitute for that from the organism. About 100 cc. of a normal solution of sulfuric acid were poured into the chamber in which the organisms had been kept, and into this were dripped at a constant rate solutions of sodium bicarbonate of different but known concentrations. The rates of change in the indicator did not correspond to the calculated rates of liberation of carbon dioxide from the solutions, and it soon becomes apparent that all the carbon dioxide produced was not liberated but that part of it remained behind in the solution. The dripping of weak acid on dry sodium bicarbonate or on marble also failed to yield consistent results.

The method of calibration finally adopted was that of making mixtures of carbon dioxide and atmospheric air and of introducing them at a known rate into the apparatus in place of the organism. Into a narrow graduated glass receiver filled with water carbon dioxide was bubbled till a given volume at atmospheric pressure was attained, whereupon, the remainder of the receiver was quickly filled with atmospheric air and the whole closed. This operation was carried on over a water bath so quickly that the solution of the carbon dioxide in the water was reduced to a negligible amount. Mixtures of gases containing 0.4, 1, 2, and 4 per cent of carbon dioxide were used. Each of these mixtures was introduced through a connecting tube into the chamber of the apparatus in which the organism had been kept by running into the gas receiver at a known rate a minute stream of mercury and thus driving out the mixture of gas. As the glass gas receiver was graduated, the amount of gas driven out of it in any given time could be easily read off by observing the levels at which the mercury stood in it as shown by the scale on its wall. Thus a constant and measurable supply of carbon dioxide was substituted for that produced by the organism. In this form of procedure the rate of change in the indicator corresponded very closely to the rate of introduction of carbon dioxide. Since the volume of mixed gases introduced into the apparatus in any test was very small in comparison with the total volume of the apparatus,

no attention was given to the slight increase of density that must have ensued in the course of a test by introducing gas into an apparatus already filled with air at atmospheric pressure.

The indicator used in these tests was an aqueous solution of phenolsulfonephthalein (Hynson, Westcott, and Dunning), and the times in seconds necessary to change its tint from that characteristic for pH 7.78 to 7.36 (Osterhout and Haas, 1918), at the four concentrations of carbon dioxide used, are given in Table I.

The steps necessary to determine the amount of pure carbon dioxide in ten-thousandths of a milligram delivered per second to

TABLE I.

Times in Seconds Needed to Change a Standard Solution of Indicator (Phenolsulfonephthalein) from pH 7.78 to 7.36 by the Introduction into the Osterhout Respiratory Apparatus of Four Mixtures of Gases Containing Respectively 0.4, 1, 2, and 4 Per Cent of Carbon Dioxide.

Concentration of carbon dioxide, per cent.....	0.4	1	2	4
Time to change indicator from pH 7.78 to 7.36, *sec*.....	569	218	106	58
	559	228	104	58
	563	214	106	56
	558	221	108	58
	564	229	106	57
Average time.....	562.6	222.0	106.0	57.4
Rate $\left(\frac{1}{\text{average time}}\right)$.....	0.00178−	0.00450+	0.00943+	0.0174+

the apparatus are shown in Table II. In the table are given: (A) the percentage concentration of the carbon dioxide mixture, followed by the time (B) required to deliver 10 cc. of this mixture to the apparatus. By dividing 10 cc. by the number of seconds needed to deliver that amount of gas to the apparatus, the volume of gas delivered per second was found (C). By multiplying this volume by the appropriate per cent indicating the proportion of the impure carbon dioxide contained in the given mixture, the several volumes of impure carbon dioxide delivered were determined (D). By absorbing with sodium hydroxide in a graduated tube a sample of

the carbon dioxide used, it was found that this gas was pure to the extent of 97.2 per cent and on introducing this correction into the calculation, the volume of pure carbon dioxide delivered per second

TABLE II.

Observed Times in Seconds for the Delivery of 10 Cc. of the Four Gas Mixtures Containing Respectively 0.4, 1, 2, and 4 Per Cent of Impure Carbon Dioxide (B), Calculated Amounts in Hundred-Thousandths of a Milligram of Pure Carbon Dioxide Delivered per Second to the Apparatus (F), and Calculated Constants for the Apparatus (G).

A. Impure carbon dioxide, *per cent*	0.4	1	2	4
B. Observed times for delivery of 10 cc. of mixed gas, *sec.*	297	296	293	298
C. Calculated volume of gas delivered per sec. ($= \frac{10}{297}$ etc.), *cc.*	0.003367+	0.003378+	0.003413−	0.003356−
D. Calculated volume of impure carbon dioxide delivered per sec. (= C × A), *cc.*	0.00001347−	0.00003378+	0.00006826−	0.00013424−
E. Calculated volume of pure carbon dioxide delivered per sec. (= D × 0.972), *cc.*	0.00001309+	0.00003283+	0.00006635−	0.00013048+
F. Calculated weight (in hundred-thousandths of a milligram) of pure carbon dioxide delivered per sec. (= E × 1.75984 × 100,000)	2.304−	5.777+	11.676+	22.962+
G. Constants for apparatus (= Average time, Table I, × F.)	1,296+	1,282+	1,238−	1,318+

in each of the four tests was calculated (E). To change the quantitative determinations of carbon dioxide from volumes to weights, the volumes of this gas in cubic centimeters were multiplied by 1.75984,

the weight in milligrams of 1 cc. of carbon dioxide at 24°C. and 762 mm. of barometric pressure, the conditions of the test. The result of this operation was then multiplied by 100,000 to permit the final number to be expressed in hundred-thousandths of a milligram (F). In this way the weight of carbon dioxide delivered per second and expressed in hundred-thousandths of a milligram was arrived at.

In the use of this apparatus it is assumed that equal concentrations of carbon dioxide produced the same color tints in the indicator. Since in all four tests the readings were always begun at the same tint and ended at another tint always the same, it follows that the amount of carbon dioxide delivered per second in each test multiplied by the number of seconds over which the test extended ought to yield a constant. And such seems to be the case, as is shown in the last line of Table II in which the product of the average times (Table I) by the weights of carbon dioxide delivered per second are given. These constants vary from 1,238− to 1,318+ and average 1,283.5; they are a measure of the amount of carbon dioxide necessary in this particular piece of apparatus to change the indicator from the tint characteristic of pH 7.78 to that for pH 7.36.

Another way of expressing these relations is shown in the graph (Fig. 1) in which the weights in hundred-thousandths of a milligram of carbon dioxide delivered are plotted as abscissæ, and the rates at which the indicator changed as ordinates. As the plotting shows, the relations are clearly linear.

In using these results to determine the absolute amount of carbon dioxide produced by an organism, it is convenient to express them in the form of an equation thus:

$$K = T \times W$$

where K is the constant already determined for the apparatus, T the time in seconds for the change in the indicator from one pH value to the other, and W the weight of carbon dioxide in hundred-thousandths of a milligram delivered per second. As the constant and the time in the operation described are the known factors and the weight the desired one, the most convenient statement of the equation is

$$\frac{K}{T} = W$$

and it is in this form that I have used it in calculating the weights of carbon dioxide produced by sea anemones. Thus in one instance a sea anemone weighing 0.5 gm. brought about the necessary color change in the indicator in 424.8 seconds. This animal must, therefore, have produced 1,283.5/424.8 or 3.0+ hundred-thousandths of a milligram of carbon dioxide per second. Another one also weighing 0.5 gm. brought about the same change in 420.4 seconds and by a similar calculation can be shown to have produced 3.1 − hundred-

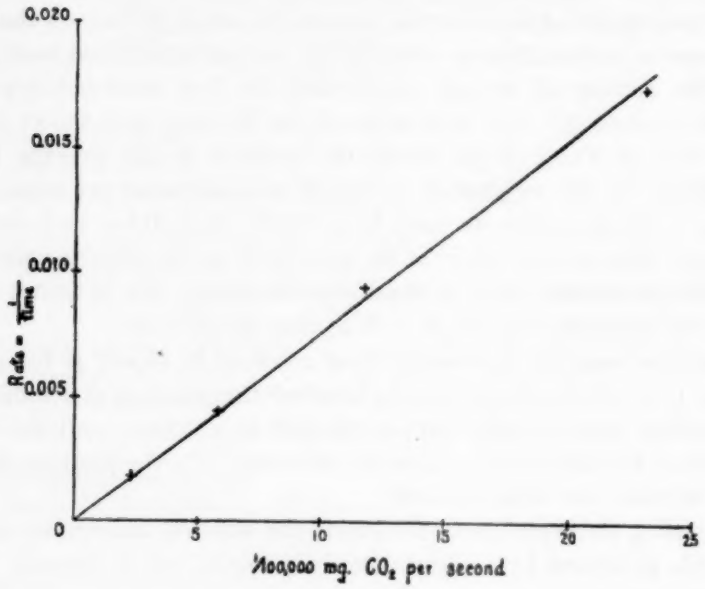

FIG. 1.

thousandths of a milligram of carbon dioxide per second. The two animals together changed the indicator over the requisite range in 213 seconds which when used as a basis of calculation yield 6.0+ hundred-thousandths of a milligram of carbon dioxide per second or almost exactly twice that of the sea anemones taken separately. Thus the proposed formula affords an easy means of calculating the absolute amount of carbon dioxide excreted when the time of the indicator change and the apparatus constant are known.

It must be clear from the foregoing account that each apparatus will have its own constant, and that anyone who wishes to calibrate such an apparatus for the absolute amount of carbon dioxide produced will first have to determine this constant by some such method as that already described. Once, however, having made this determination, the formula already given may be used with perfect accuracy for the calculation of the absolute amount of carbon dioxide produced. Since the constant is a measure of the amount of carbon dioxide necessarily present in a given piece of apparatus, in order that a standard change in the indicator shall take place, and since this amount is spread through the space contained within the apparatus, it is probable that the constant is directly related to the volume of the apparatus and will be small in an outfit of small volume and large in one of large volume, but such relations have not as yet been worked out.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

I. The Solubility of Certain Proteins at Their Isoelectric Points.

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In 1899 W. B. Hardy observed that "under the influence of a constant current the particles of proteid in a boiled solution of egg white move with the negative stream if the reaction of the fluid is alkaline; with the positive stream if the reaction is acid" (1). The protein in the "boiled solution of egg white" had, of course, been denatured, but Pauli (2, 3) later investigated the direction of migration of undenatured serum albumin in an electric field, and found that it, too, moved toward the cathode in an alkaline solution and toward the anode in an acid solution. Presumably the charge on the protein was negative in alkaline, but positive in acid solution.

Michaelis was the first investigator to determine the hydrogen ion concentration at which the migration of a protein changed its direction. He studied serum albumin, and found that the change was abrupt (4). It occurred within exceedingly narrow limits. At a hydrogen ion concentration of 2.1×10^{-5} serum albumin migrated toward the cathode. At smaller hydrogen ion concentrations than 1.9×10^{-5} it migrated toward the anode. At 2.0×10^{-5} the protein appeared to be in an isoelectric condition (5) and this hydrogen ion concentration was accordingly termed the isoelectric point.

The movement in an electric field of particles charged with respect to their surrounding medium is termed cataphoresis. From cataphoresis the sign, and the average magnitude, of the charge can be determined, but not its nature or origin. The nature of the charge of the protein molecule will be discussed in a later section of this paper from a theoretical point of view.

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The isoelectric points of many proteins have been inferred from the change in the direction of their migration in an electric field. The method of cataphoresis has, however, not been found universally applicable. Certain proteins, notably glutenin, casein, and the prolamines are only very slightly soluble at certain hydrogen ion concentrations. If acid, or, under other circumstances, alkali, be added to their solutions a precipitate appears, increases in amount, and finally disappears if the hydrogen ion concentration is sufficiently altered. Michaelis observed (6) that such proteins, like the denatured albumin that Hardy first studied (1), migrated to the cathode on the alkaline side of the precipitation zone, and to the anode on the acid side. Accordingly he concluded, in part from theoretical considerations to be discussed later, that the protein precipitated in the isoelectric condition, and that the maximum precipitation coincided with the isoelectric point. The phenomenon of precipitation has therefore generally been substituted for the phenomenon of cataphoresis in determining the isoelectric point of this class of substances; the isoelectric point being considered coincident with the point of maximum precipitation or flocculation.

Still a third class of proteins, the globulins, although like the last group they are also precipitated at certain hydrogen ion concentrations in the absence of any appreciable concentration of electrolytes, are readily soluble in solutions of neutral salts. In this class also the precipitation zone has been found to correspond to the isoelectric zone. That is to say, isoelectric globulin was found to migrate toward the cathode when dissolved by acid, toward the anode when dissolved by base. When serum globulin was dissolved in a salt solution, however, neither Michaelis (6) nor Chick (7), who confirmed his observations, was able to detect a charge on the protein molecules by cataphoresis.

Since globulins are dissolved by neutral salts over a considerable range of hydrogen ion concentrations—and the nature of this phenomenon and its relationship to the isoelectric point of the globulins we shall reserve for a subsequent communication—practise has consisted in reducing this range by the removal of salt, and then either in noting the point of maximum precipitation of the globulin, or the limiting hydrogen ion concentrations at which migration occurred in an electric field.

Experience has shown that neither of these methods of determining the isoelectric point of a globulin is altogether satisfactory. It is true that the precipitation of a globulin passes through a sharper maximum the freer it is from salt. Freedom from salt, at least as applied to the proteins is, however, relative. Probably no protein has even been prepared that was completely "ash-free." Moreover, three distinct sources of error may make, and in the experience of the writer have made the point of maximum precipitation of a protein appear at a hydrogen ion concentration other than its isoelectric point.

The first of these is the presence of another protein with a slightly different isoelectric point. The observed precipitation is in this case the sum of the precipitations of the two proteins. As a result the zone is usually widened, and the point of maximum precipitation shifted in the direction of the isoelectric point of the second protein. The magnitude of the shift, and therefore of the error, depends upon the difference in the isoelectric points of the two proteins, upon their relative concentrations, and upon their relative solubilities.

The presence of salts containing either bivalent or trivalent cations or anions may also lead to error. For multivalent ions, even in low concentration, shift the point of maximum precipitation to a hydrogen ion concentration other than the isoelectric point of the protein. Hardy (8), Mellanby (9), Osborne and Harris (10), and later Hopkins and Savory (11), have shown that the solvent action of salts upon globulins increases greatly with the valence of their ions. Moreover, cations of high valence are more effective in dissolving globulins on one side of the isoelectric point and anions on the other. For this reason the salts of monovalent acids and bivalent bases, (or the salts of monovalent bases and bivalent acids) exert a greater solvent action upon globulins at certain hydrogen ion concentrations than at others. This also results in a shift in the precipitation zone.

In greater concentration salts precipitate proteins of all classes. The precipitating action of electrolytes, like the solvent action, increases greatly with the valence (1). Michaelis has recently shown theoretically and experimentally how the presence of salts of high valence shifts the point of maximum precipitation of proteins (12).

Finally, I have observed that even uni-univalent salts of the type of NaCl in relatively low concentrations, may shift the precipitation

(though possibly not the flocculation) and therefore the apparent isoelectric point of tuberin and serum globulin to a slight degree. The reason for this appears from a theoretical consideration of the nature of the charge on the protein molecule.[1]

As a result of the unsatisfactory nature of the experimental methods that have been employed in determining the isoelectric point of the slightly soluble proteins, and in view of the manifest importance and significance of the isoelectric point for the interpretation of other aspects of the physical chemistry of the proteins, it seems necessary to base these studies upon better criteria of the identity of the proteins under investigation.

Theoretical.

Hardy had been led to examine the cataphoresis of denatured albumin from a consideration of the contemporary conceptions of colloidal chemistry. Picton and Linder (15) had "established . . . . that the direction of the movement of colloidal particles under the influence of an electric current is determined by their chemical nature," and Hardy concluded[2] that "proteid molecules seem therefore to act as basic or acid particles according to the circumstances in which they find themselves."

In the same year in which Hardy published these conclusions Bredig (16) extended the theory of electrolytic dissociation to the case of molecules that act both as acids and as bases; that is, to amphoteric electrolytes. The amphoteric nature of the amino-acids and also of the proteins had already been recognized (17, 18, 19).

[1] If the assumption be made that the charge on the protein is due to its dissociation as an amphoteric electrolyte, then the cation of any salt will depress the dissociation of any protein through its common ion on the alkaline side of the isoelectric point, and the anion on the acid side. Sörensen (13) has used this phenomenon as a method of determining the isoelectric point of egg albumin; the isoelectric point coinciding with the hydrogen ion concentration that is unchanged by the addition of a neutral salt. I have attempted to apply the method to tuberin (14) but it is not entirely suitable to the globulins. Moreover, even where the method is applicable for the determination of the isoelectric point in this way, the effect of a common ion in depressing dissociation and thereby affecting solubility need not be identical on both sides of the isoelectric point.

[2] Hardy (1), p. 297.

Because of their simpler structure, the amino-acids have been found more suitable than the multivalent proteins for the experimental verification of dissociation theory. Accordingly the generalizations, in terms of which we have attempted to explain the dissociation of the proteins, were first worked out for their prototypes, the amino-acids.

Amino-acids possess at least one amino group, and at least one carboxyl group. The amino groups dissociate as bases and combine with acids, much as does ammonia. The carboxyl groups dissociate after the manner of organic acids and combine with bases. As a result of its amphoteric nature an amino-acid can form internal salts (20, 21, 22, 23). If the amino group of one acid combines with the carboxyl group of the next with the loss of a molecule of water, a dipeptide is formed which in turn is an amphoteric substance. Polypeptides, in which many amino-acids are combined in this manner, have been synthesized by Emil Fischer (24). They simulate proteins in behavior, and suggest that the free valences in the protein molecule are in all probability derived at least in part from the free groups of the amino-acids.

The strength of these groups in a number of amino-acids was first determined by Winkelblech (20) in 1901, from the hydrolysis of their acid and basic salts. In the hands of Walker (21), however, Winkelblech's data revealed the relations that obtain between the degree of dissociation of an amphoteric electrolyte and its strength as an acid and as a base. Following Walker in the main, therefore, and in part the more recent investigators[3] who have amplified his conceptions, we shall deduce the fundamental equations for the dissociation of an amphoteric substance, for our present purposes a protein, P.

Let HPOH represent the undissociated protein molecule. The protein can dissociate into an hydrogen ion and a protein anion,

$$(HPOH) \rightleftarrows (H^+)\ (POH^-) \tag{1}$$

and into an hydroxyl ion and a protein cation,

$$(HPOH) \rightleftarrows (HP^+)\ (OH^-) \tag{2}$$

[3] Notably Hardy (1), Sörensen (13), Lundén (23), Michaelis (25), Henderson (26), Robertson (27), Pauli (28), and Loeb (29).

if the protein be considered as a monovalent acid, giving rise to but one hydrogen ion, and a monovalent base, giving rise to but one hydroxyl ion. That the multivalent protein can under certain restricted circumstances be considered as uni-univalent we shall presently show.

The mass law equation defining the dissociation of the protein as an acid may be written

$$\frac{(H^+)\ (POH^-)}{(HPOH)} = Ka \tag{3}$$

and as a base

$$\frac{(HP^+)\ (OH^-)}{(HPOH)} = \frac{(HP^+)\ Kw}{(H^+)\ (HPOH)} = Kb \tag{4}$$[4]

Equations (3) and (4) on multiplication yield an expression for the dissociation of the pure ampholyte, or rather for the square of the dissociation

$$\frac{(HP^+)\ (POH^-)}{(HPOH)\ (HPOH)} = \frac{Ka \cdot Kb}{Kw} \tag{5}$$

The method of deriving this equation involves the elimination of the hydrogen ion concentration from both equations (3) and (4). As a result the pure ampholyte can exist at only one hydrogen ion concentration; namely, that at which the ampholyte dissociates to form as many anions as cations. This point has been identified as the point at which the migration of protein in an electric field changes in direction; that is, as the so called isoelectric point. It has been defined by Michaelis as the point at which

$$(HP^+) = (POH^-) \tag{6}$$

and is determined by substituting in equation (6) the value of (POH^-) in equation (3) and of (HP^+) in equation (4). We then obtain

$$\frac{Ka\ (HPOH)}{Kb\ (HPOH)} = \frac{(H^+)^2}{Kw} \tag{7}$$

and if we assume that the undissociated protein molecule is the same on both sides of the isoelectric point (30) we obtain

$$\frac{Ka}{Kb} = \frac{(H^+)^2}{Kw} \tag{8}$$

[4] $(H^+)\ (OH^-) = Kw$

At the hydrogen ion concentration that corresponds to the isoelectric point a definite ratio obtains between the total amount of the acid dissociation and the total amount of the basic dissociation. This ratio obtains irrespective of how many acid valences of different strength or how many basic valences of different strength are involved. For at any one hydrogen ion concentration the number of active acid constants may be considered equal to one constant, Ka, and the active basic constants to another constant, Kb (31). For this reason the multivalent protein may be treated as a simple ampholyte at its isoelectric point. We shall so consider it in this paper, since we are, for the moment, only concerned with the solubility of certain proteins at their isoelectric points.

Michaelis pointed out that the precipitation of a protein was at a maximum at its isoelectric point, since dissociation was at a minimum (25). This follows from equation (5) if we define maximum precipitation as minimum solubility, and assume that protein is as a rule more soluble in the dissociated than in the undissociated state. According to this conception a protein should be more soluble the greater its amphoteric constants. I have shown (31) that this was the case for different classes of proteins in a previous communication, by using the acid- and the base-combining capacity of a protein as a measure of its amphoteric strength.[5]

In order to pursue this investigation further it was necessary to correlate the solubility of proteins with their dissociation. This involved accurately determining the solubility of a number of proteins at their isoelectric points, and quantitatively distinguishing between the concentrations of dissociated and undissociated protein. Let the solubility of a protein, P, be S. This solubility is made up of the concentration of the undissociated protein molecule and of the dissociated protein ions, in the saturated solution. We may write

$$S = (HPOH) + (HP^{+}) + (POH^{-}) \qquad (9)$$

[5] In practise this was estimated by titrating electrometrically 1 gm. of protein with NaOH and HCl and measuring the rate of change of the hydrogen ion concentration (by the slope of the tangent to the titration curve) at the isoelectric point. These estimates are therefore subject to revision when sufficiently accurate data of the molecular weights of the proteins are available.

provided the protein is quite uncombined with acid or with base. If a part of the protein is combined with a base B, however, or a part with an acid A, we must write[6]

$$S = (HPOH) + (HP^+) + (POH^-) + (BP) + (PA) \qquad (10)$$

But let us first consider the case of an uncombined protein at its isoelectric point. Its degree of dissociation is given by equation (5) as equal to $\sqrt{\frac{Ka \cdot Kb}{Kw}}$. This ratio is, as we have shown above, a constant for any protein at its isoelectric point. Walker showed that the degree of dissociation of an amino-acid as acid or as base, $\frac{(HP^+)\,(POH^-)}{(HPOH)^2}$, was a constant, independent of dilution from conductivity data.

In the case of a protein that is relatively insoluble at its isoelectric point the undissociated protein HPOH may be assumed to have a definite solubility. Moreover, this molecule dissociates, as we have seen, to form a protein cation HP^+, and a protein anion POH^-. The solubility determined experimentally must be the sum of the concentrations of the undissociated protein molecule and of the protein ions. But if the concentration of the undissociated molecule is constant, the degree of dissociation must also be constant at the isoelectric point. Solubility must therefore be constant so long as the solution is saturated with respect to undissociated protein.

Experiments now to be described have shown that this is the case when the protein is uncombined with base or acid. The solubility of casein and of the two globulins, tuberin and serum globulin, that have thus far been investigated, was found to be constant at the respective isoelectric points of the proteins, when the amount of protein precipitate with which the solution was in heterogeneous equilibrium was varied within wide limits.[7]

Only at the isoelectric point was solubility independent of the amount of the protein in the system. At greater hydrogen ion concen-

[6] I am indebted to Prof. S. P. L. Sörensen for first calling my attention to the convenience of treating solubility as an unknown function of concentration.

[7] If the amount of the protein precipitate was increased beyond a certain point, a secondary effect slightly increasing solubility was detected.

CORRECTION.

On page 704, Vol. iv, No. 6, for $\frac{(HP^+)\ (POH^-)}{(HPOH)}$ read $\frac{(HP^+)\,(POH^-)}{(HPOH)^2}$.

trations the protein is combined with acid, and at smaller with base.[8] In either case the solubility is no longer equal to the sum of the undissociated protein molecule and its resultant ions, but to the sum of these and the protein acid or basic compound. Equation (10) and not equation (9) then obtains. Moreover, the degree of dissociation of the compound of a protein with either a strong acid or a strong base must be very great in comparison with the dissociation of the pure protein, for the same reason that the dissociation of the salt of any weak electrolyte and a strong electrolyte is very much greater than the dissociation of the weak electrolytes (32, 33). As a result, when the protein is combined with even a very small amount of either acid or base, the solubility due to the dissociation of the protein must be considered very small in comparison with the solubility due to the dissociation of the protein compound. But the concentration of the protein compound is not independent, but is a function, of dilution, as these experiments and previous ones have indicated (34, 35).

These observations, therefore, present a new method of determining the isoelectric point of a protein. This method is free from the errors that often enter into the determination of the minimum in solubility of a protein, though theoretically, and in practise, the two methods are capable of giving the same result. But, since the presence of a foreign protein (unless it has the same solubility and the same isoelectric point), or of multivalent cations or anions, or of a neutral salt, may shift the point of minimum solubility of a protein in the manner and for the reasons that have already been described, the presence of these impurities renders impossible the determination of the true isoelectric point. Upon their removal, however, protein will dissolve in water to a constant and characteristic extent, and will dissociate to a constant and characteristic extent. The hydrogen ion concentration due to this dissociation is also a characteristic of each protein, and has come to be known, as we have seen, as its isoelectric point.[9]

[8] Experiments that will be reported in a subsequent communication suggest that a protein can exist in combination with acid and base even at its isoelectric point.

[9] In practise the hydrogen ion concentration due to the dissociation of pure protein is determined with great difficulty because of its very small solubility.

For all these reasons we have come to consider the solubility of a protein at its isoelectric point as a fundamental physicochemical constant, characterizing and identifying the protein under investigation. We will attempt in subsequent communications to relate this constant to the amphoteric dissociation of the proteins.

EXPERIMENTAL.

The Purification of Protein.

The three proteins whose isoelectric solubilities have thus far been determined, serum globulin, tuberin, and casein, were each purified as far as possible from other proteins, from multivalent anions and cations, and from all but the last trace of electrolytes, by special methods adapted to the nature of each. They were first prepared either as ammonium or as sodium compounds. Analyses were then made of the amount of ammonia or of sodium in these compounds, and the amount of hydrochloric acid required to neutralize the base and precipitate the protein at its isoelectric point[10] was calculated.

The acid used was usually 0.01 N hydrochloric acid, and was delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly mixed by a motor-driven glass screw-shaped stirrer which constantly forced fresh portions of the protein past the glass tip from which the acid was being delivered, in a manner similar to that described by Baker and Van Slyke (37). In this way the protein was never exposed to the denaturing effect of a local excess of acid. After precipitation had begun, the process of neutralization was further retarded in order to allow new states of equilibrium to be fully attained. As the end was approached small samples were removed, and the hydrogen ion concentration electrometrically determined.

The isoelectric reaction was usually reached, at least in the case of serum globulin, before the calculated amount of base had been neutralized. The base in

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The measurements that have been made thus far suggest that when the hydrogen ion concentration of such a protein solution varied it moved in the direction of neutrality.

[10] Loeb has recently discussed the significance of the isoelectric point for the purification of protein (36).

these cases was ammonia, and was determined in the manner described by Sörensen (13), in the filtrate and washings from globulin coagulated by heat at its isoelectric point. It is therefore possible that the very small excess of ammonia reported was due in part, or in whole, to the splitting of ammonia from the protein molecule. That this is involved in the coagulation of proteins by heat is rendered probable by the investigations of Sörensen and Jürgensen (38) on albumins, and by the observation that such proteins as casein and glutenin, which are very slightly soluble at their isoelectric points are not heat-coagulable. The alternative possibility is that serum globulin is capable of combining with a certain amount of base even at its isoelectric point. This will be the subject of another communication. In Table I are collected the ammonia concentrations in the serum globulin preparations that have been studied thus far, and also the hydrochloric acid that was required to bring them to the isoelectric point.

TABLE I.

Analyses of Serum Globulin Preparations.

Preparation of serum globulin.	Fraction.	Protein.	pH^+	NH_3 in preparation.	HCl used to bring protein to isoelectric point.	Excess ammonia ($NH_3 - HCl$).	Excess ammonia $\left(\frac{NH_3 - HCl}{NH_3}\right)$.
		gm.		*mols*	*mols*	*mols*	*per cent*
I*	No fractionation.		7.0	0.0014	0.0010	0.0004	28.6
II*	Pseudoglobulin.		7.0	0.0038	0.0029	0.0009	23.7
IVa	"	34.15	6.9	0.0051	0.0040	0.0011	27.5
IVb	Euglobulin.	14.73	7.4	0.0029	0.0023	0.0006	26.1
Va	Pseudoglobulin.	32.13	7.1	0.0035	0.0025	0.0010	28.6

* Serum Globulins I and II were prepared in collaboration with Professor Sörensen at the Carlsberg Laboratorium in Copenhagen, early in 1920. They were prepared as ammonium compounds and were not brought to the isoelectric point. The data that are given are derived from certain experiments in which the protein was brought to the isoelectric point in order to study the solvent action of neutral salts. The method of further purifying globulin at its isoelectric point was subsequently devised.

Stirring was always continued for many hours after the isoelectric point was reached, in part to break up the flocks or aggregates that form under these conditions, and to prevent the occlusion of impurities in them, in part to hasten the attainment of equilibrium in so sluggish a system. Occasionally it has been found necessary to break the larger aggregates with a pestle and mortar. The protein was finally returned to the cold room, from which it was never removed for

more than a few hours, and the precipitate allowed to settle. The water-clear supernatant liquid was then decanted; distilled water added approximately to the same volume; and the precipitate again stirred for several hours. This operation was usually repeated from six to fifteen times, and was only stopped when the wash water no longer contained chloride, and when the amount of protein dissolving in the wash water had become constant.

The course of the purification of the euglobulin fraction of preparation IV, as measured by the protein nitrogen in an aliquot of successive washings, is tabulated below. Approximately 14 gm. of this material (14.72 gm. before neutralization) were triturated each day with distilled water. The total volume was 350 cc., and about 190 cc., or slightly more than half of the clear supernatant liquid was decanted each morning. It will be noted that at first the concentration of

TABLE II.

Purification of Serum Globulin IV b.

Wash water No.	1	2	3	4	5	6	7
Nitrogen in 10 cc. of wash water, *mg.*	7.60	3.64	1.86	1.40	0.94	0.80	0.63

soluble protein was also approximately halved each day, but that as the purification proceeded, it approached a constant value.

It is apparent that this method of purification is only applicable to relatively insoluble proteins, since the loss of material would otherwise be prohibitive. In the case of relatively insoluble proteins, however, it has the advantage of ensuring the identity of the product. For unless two proteins have the same isoelectric point, and very nearly the same solubility, this procedure may be used to effect their complete separation. The more soluble protein dissolves in the successive wash waters, and can be recovered from them by concentrating in dialyzers, under negative pressure. It will, however, still contain a trace of the less soluble protein.

Recourse was first had to this method in the preparation of the pseudoglobulin fraction of serum IV. When this fraction was rendered isoelectric a large amount of protein was at first found in the

wash water.[11] This was for the most part globulin like the rest, dissolved by the ammonium chloride formed in the neutralization of the ammonium globulinate by hydrochloric acid. The wash waters containing this protein were accordingly dialyzed further and concentrated.

When the isoelectric protein had finally ceased to give off impurities and readily soluble protein, it was suspended in the desired volume, and aliquot parts used for analyses. It has been found impracticable to dry proteins, since it is almost impossible uniformly to wet their surface after they have been dried by alcohol and ether. As a result, there can be no guarantee that a true equilibrium is subsequently reached between all of the protein and its solvent. Moreover, it has been thought preferable, for the purposes of this investigation, to retain any lipoid that might be in fixed combination with our proteins, rather than risk the danger of denaturing them by alcohol or ether.

The Measurement of Solubility.

In order to obtain reproducible measurements of the solubility of a protein a great many precautions must be observed. To saturate the solution with protein it is necessary to bring the heavy flocculent precipitate into intimate contact with the solvent for a long period of time. This can only be accomplished by protracted mechanical agitation, since the diffusion velocities of the proteins are exceedingly low because of their large molecular weights. If the protein precipitate were not enormously subdivided, and not brought into intimate contact with every part of the solvent, the latter would remain unsaturated.[12] If the ordinary methods of agitation are used, however, the solution foams, its concentration changes, and a portion of the protein is likely to become denatured. Moreover, soft glass containers cannot be used, since in them the protein combines with enough alkali to increase its solubility appreciably.

[11] The isoelectric solubility in my earlier preparations of serum globulin, which had not been washed at the isoelectric point, was as high as 75 per cent of the total protein present.

[12] Under these circumstances the solvent may enter the protein-rich phase, because of its greater diffusion velocity, and give the appearance of swelling.

Our practise has been to pipette an aliquot part of a preparation into a Pyrex volumetric flask of from 25 cc. to 100 cc. capacity. When this was at first attempted it was found that the error due to sampling the suspension of a flocculent protein precipitate was frequently as

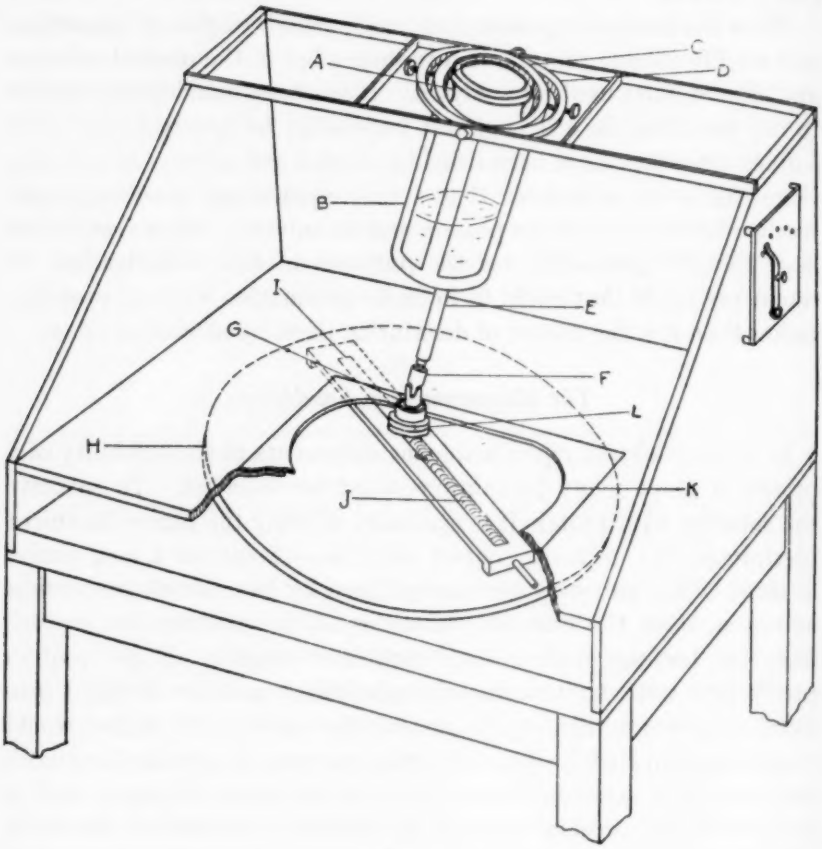

FIG. 1.

great as 3, or even 5 per cent. Accordingly an apparatus was devised by means of which the protein precipitate could be maintained in a state of fine subdivision during pipetting (Fig. 1).

This was accomplished by placing the bottle (*A*) containing the protein in a carriage (*B*) hung, as is a compass, in concentric rings (*C*) and (*D*). As a result,

the neck of the wide mouthed bottle that is used is always in the same position, and a pipette may be inserted and a sample withdrawn even when the bottle is performing more than a revolution a second. The base of the carriage is attached through a sliding valve (*E*) and a universal joint (*F*) to an eccentric (*G*) on a driving wheel (*H*). Instead of being fixed at a definite distance from the centre of the wheel, (in which case the motion of the bottle would be circular) the eccentric moves freely on ball-bearings in a slot (*I*) but is forced by a spring (*J*) to impinge on a track (*K*) with which it makes contact through a roller-bearing (*L*). The shape of the track alone determines the motion of the carriage. An ovoidal shape has been adopted in order at once to prevent foaming and centrifugal action. With the aid of this apparatus we have been able to sample protein suspensions with an accuracy of 0.3 per cent.

After the protein had been delivered into the volumetric flasks the solvent was slowly added, drop by drop, from a burette until it rose to the graduated mark on the neck of the bottle. In certain experiments, the space above was further reduced by the addition of glass beads of a grade that gave off no measurable alkali, and by a drop of toluene added to prevent bacterial action. The flasks were finally stoppered, fixed in place in the carriage of a specially designed shaking machine (Fig. 2), and shaken in a water thermostat at 25.0° ± 0.1°C. While the shaking machine was in motion, the necks of the flasks were covered with inverted test-tubes to prevent contamination from the spray of the bath.

As the carriage went back and forth on tracks in the water bath, the glass beads in the small volumetric flasks functioned as a very efficient ball mill and kept the protein precipitate mechanically suspended. In our experience the heavy flocculent isoelectric protein has usually been brought into equilibrium with its solvent in from 24 to 72 hours. The shaking machine was then stopped, the contents of the flasks allowed to settle slightly, and the saturated solutions filtered on No. 42 Whatman filter papers. By means of a water jacket through which the water of the thermostat was kept in constant circulation by a rotary pump, the filtration was carried on at nearly the same temperature as the bath. At least 8 cc. of the protein solution was always used to wash the filter paper, and was accordingly discarded. Aliquot parts of the remainder of the filtrate were measured out with carefully calibrated pipettes into Kjehldahl flasks. Analyses were usually made in triplicate. 20 cc. of sulfuric acid

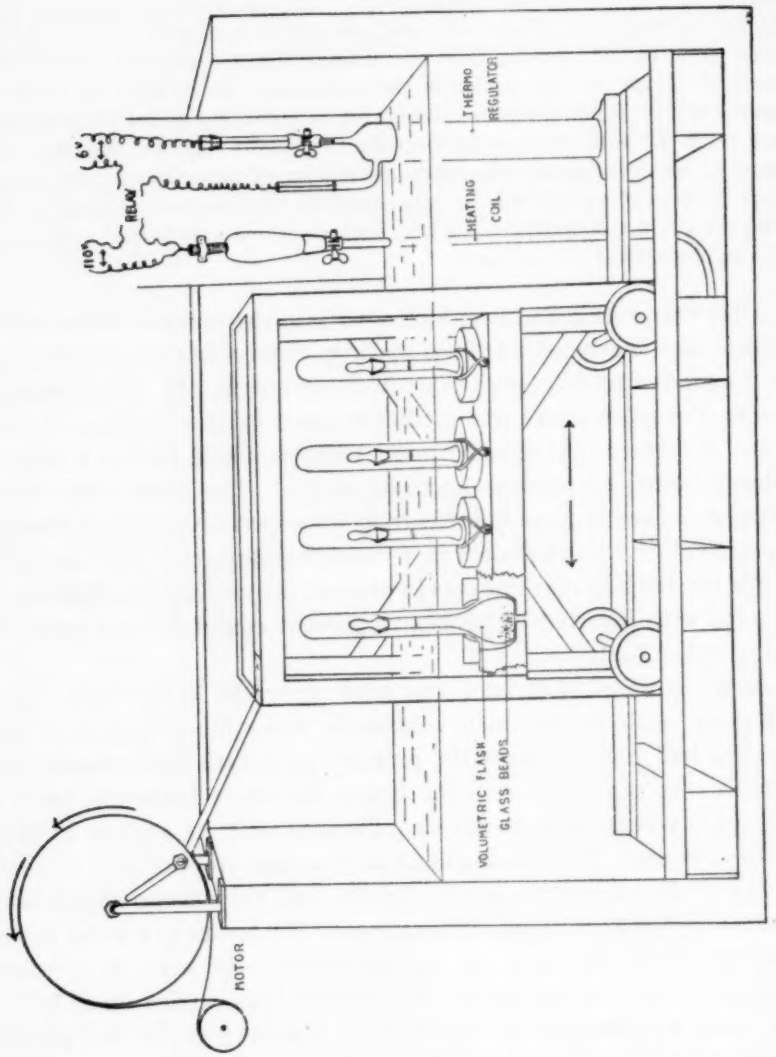
THERMO REGULATOR
HEATING COIL
RELAY
110v
VOLUMETRIC FLASK
GLASS BEADS
MOTOR

FIG. 2.

(Baker and Adamson: C. P.), 5 gm. of potassium sulfate and 0.7 gm. of mercuric oxide were added, and the protein was digested. When digestion was complete, the colorless solution was cooled and diluted to about 250 cc. with distilled water. 1 gm. of sodium hypophosphite was then added to reduce the mercuric oxide, and 75 cc. of saturated sodium hydroxide were added to neutralize the sulfuric acid and liberate the ammonia. The ammonia was then distilled into a measured amount of N/7 hydrochloric acid. The excess of acid together with a blank containing the same amount of acid were then titrated with N/14 sodium hydroxide, a combination of methylene blue and methyl red being used as indicator (39). Sodium hydroxide of this strength was used, since each cc. required to neutralize the blank in excess of that required to neutralize the unknown then corresponds to 1 mg. of nitrogen, (atomic weight of N = 14.01).

The Solubility of Serum Globulin.

Fig. 2.

Serum globulin was the first protein that we purified from other proteins, from acids, bases, and salts, and from its own soluble compounds to a sufficient extent to obtain a product of constant solubility,—a product that would dissolve in water to a constant extent. The results of five experiments are recorded in Table III. They are in striking contrast to what they would have been had a second protein, or a soluble form of the same protein, been present. A twentyfold variation in the amount of suspended protein produced no commensurate change in solubility, though there was a very slight increase in solubility with an increase in the amount of the preparation. The solubilities recorded are the average of three analyses. They indicate that serum globulin prepared in this way only dissolves in 1 liter of water at 25°C. to the extent of approximately 0.1 gm.

Serum globulin has now been prepared in this manner a second time, and several further precautions taken both in its purification, and in the determination of its solubility. The globulin in the first preparation (IV) was derived from citrated plasma of the cow, in the second from cow serum. In both preparations the so called pseudoglobulin fraction was used. This fraction is supposedly freer from lipoid (40) and from phosphorus (40, 41) than is euglobulin. Whether

or not these are the same or different proteins we shall consider at another time from the point of view of their respective solubilities.

The pseudoglobulin was freed from euglobulin by repeated fractional precipitation with ammonium sulfate. The euglobulin is supposed to be completely precipitated from a solution that is one-third saturated with respect to ammonium sulfate, and the pseudoglobulin from a solution that is one-half saturated. In practise it was found necessary to adopt the procedure described by Haslam (41). Both precipitates were collected, redissolved, and again one-third saturated with ammonium sulfate. The filtrate from the reprecipitated euglobulin was then added to the pseudoglobulin fraction, and a precipitate appearing in the pseudoglobulin fraction combined with the

TABLE III.

Solubility of Serum Globulin IVa.

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H_2O at beginning of experiment.				
		mg. 10.9	mg. 21.7	mg. 38.0	mg. 54.3	mg. 190.0
		Protein nitrogen in 25 cc. filtrate.				
	1921	mg.	mg.	mg.	mg.	mg.
6	June 13	0.37	0.56		0.56	
9	" 21			0.37		0.44

euglobulin.[13] The pseudoglobulin was reprecipitated thirteen times in preparation IVa, and nine times in preparation V. Serum globulin Va represents a smaller and possibly a better defined fraction. Howe (42) has recently presented additional evidence for the existence of two pseudoglobulin fractions (43, 44, 45); the first precipitated at approximately 0.43 saturated ammonium sulfate. In our experience this fraction represents about three-fourths of all the pseudoglobulin.

The method that has been used in preparing this globulin as an ammonium compound was first worked out in 1919–20 in collaboration with Professor S. P. L. Sörensen, at the Carlsberg Laboratorium. It involves operations of two kinds.

[13] The precipitation of proteins by electrolytes is not independent of the protein concentration.

In the first place freeing the protein from all but one salt, ammonium sulfate. In the second place freeing the protein from the sulfate ion. The first step was accomplished by reprecipitating with ammonium sulfate at different hydrogen ion concentrations. The protein was freed from anions other than the sulfate by reprecipitation with C.P. ammonium sulfate from dilute sulfuric acid solution, approximately 10^{-4}N, and from cations other than ammonia by reprecipitation from solution in dilute ammonia. The globulin was then dialyzed, isosmotically, in the manner described by Sörensen (13). A slight excess of ammonia was maintained in the dialyzers until the globulin was completely freed from sulfate. The transformation of the soluble ammonium-globulin compound into the very slightly soluble uncombined isoelectric globulin has already been described.

A somewhat different and more exact procedure was adopted in studying the isoelectric solubility of serum globulin Va. Not only were different amounts of the globulin preparation used to saturate the same volume of water, but the same precipitate was used to saturate successive additions of water. The dates are recorded on which samples were removed for analysis. On these dates a further amount of fresh water was added and saturation recommenced. The results obtained in this way were far more concordant. They are tabulated in Table IV. As in preparation IV, more protein dissolved in the flasks which contained most protein. That this was for the most part due to the fact that all of the soluble protein had not been removed, is suggested by the fact that solubility soon fell to a lower and constant value in the flasks containing the three smallest amounts of globulin.

The persistent higher solubility in the flasks containing most precipitate is probably to be ascribed to another phenomenon that need not be considered at this time.

Twenty-four nitrogen determinations, made upon the filtrates of solutions saturated with three different amounts of protein, on 4 different days, after their solubility had become constant, agree within the errors of measurement. The average of these determinations is 0.30 mg. protein nitrogen in 25 cc., or 12.0 mg. protein nitrogen in 1 liter. This result is not very different from the lower results obtained with preparation IV. If we adopt 15.85 as the percentage nitrogen in this protein the most probable solubility of the pseudoglobulin fraction of serum becomes 0.07 gm. per liter at 25°C.

Although so insoluble when uncombined it should be noted that 1 gm. of this preparation was completely dissolved by 1×10^{-4} mols. of sodium hydroxide, or by slightly less than that amount of hydrochloric acid. The compounds of serum globulin with acids and with bases are very soluble indeed. The smallest trace of a soluble compound would have appreciably increased solubility. The measure-

TABLE IV.

Solubility of Serum Globulin Va.

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H_2O at beginning of experiment.			
		mg. 35.27	mg. 70.55	mg. 176.37	mg. 352.75
		Protein nitrogen in 25 cc. filtrate.			
	1922	mg.	mg.	mg.	mg.
108	Apr. 24	0.29	[0.52	[0.72	
		0.32	0.52	0.70	
		0.29	0.50]	]	
	" 26	0.32	0.28	0.28	[0.62
		0.29	0.29	0.33	0.62
		0.32	0.27	0.31	]
	May 1	0.30	0.31	0.31	[0.49
		0.30	0.32	0.35	0.52
		0.29	0.29	0.31	0.51]
	" 3			0.31	[0.51
				0.29	0.52
				0.31	0.51]
Average.....		0.30	0.29	0.31	

ments reported must therefore have been upon the pure ampholyte, upon the undissociated molecule and its dissociated ions.

Certain proteins retain the power to dissolve in acid and alkaline solutions even after becoming denatured. Denatured globulin will not, however, dissolve in the solution of a neutral salt. No trace of denatured material was present in this preparation. It readily dissolved to a water-clear liquid in 0.1 N solution of sodium chloride.

Although solubility data suggested that no appreciable free electrolyte was present in these preparations, their ash contents were considerable. The ash constituted 0.7 per cent of preparation IV. In preparation V more careful methods of purification and precipitation reduced the ash to 0.3 per cent.

The Solubility of Tuberin.

The measurements upon tuberin, although they have been made on but one preparation, and although they are not nearly so accurate

TABLE V.

Solubility of Tuberin I.

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H_2O at beginning of experiment.		
		mg. 3.05	mg. 15.25	mg. 30.50
		Protein nitrogen in 25 cc. filtrate.		
	1922	mg.	mg.	mg.
204	Jan. 25	0.44		
	" 30	0.37	0.55	
	Feb. 9		0.55	
206	" 15			0.57*
	" 21			0.66*

* 5 cc. sample used.

as those that have been presented for serum globulin, or those that will be recorded for casein, are included for two reasons. In the first place because they establish the approximate constancy of solubility of another globulin derived from an entirely different source; in the second place because a study of "the relation between the isoelectric point of a globulin (tuberin) and its solubility and acid combining capacity in salt solution" (14) was responsible for the inception of these studies. The data obtained at that time showed the effect of even a uni-univalent salt, sodium chloride, in shifting the minimum of protein solubility. This phenomenon has already been discussed.

They were not correctly interpreted, however, since the isoelectric point was inferred to occur at a more acid reaction than now seems probable from observations of cataphoresis (46). The inapplicability of cataphoresis in determining the isoelectric point of a globulin has also been discussed.

The same methods were used in purifying tuberin after it had been extracted from the potato, and in determining its solubility, as were used with serum globulin. Tuberin has been found to be only slightly more soluble than the latter. The measurements in the three experiments recorded in Table V indicate a solubility in water of approximately 0.1 gm. in 1 liter at 25°C. Had a larger amount of material been available, and a more protracted experiment been possible, a slightly smaller value might have been obtained.

The Solubility of Casein.

Casein has previously been prepared in a state of great purity by a method very similar to the one that we have described (37). Baker and Van Slyke were able to show that if casein was very carefully precipitated at its isoelectric point, and was then triturated with distilled water, a product of very low ash could be obtained. We have used a modification of this method in the early stages of the purification. This modification depends upon the observation of Loeb (47) that if a divalent rather than a monovalent base is used to dissolve isoelectric casein, twice the normal concentration is required. As a result the hydroxyl ion concentration of casein dissolved by calcium hydroxide is very much greater than that dissolved by sodium hydroxide. According to Loeb the pH of the soluble sodium compound of casein is 7.02 and of the soluble calcium compound 10.53. Presumably divalent bases of the type of calcium hydroxide form insoluble acid salts with casein at neutral reactions.

Our practise has rested upon this phenomenon. After the casein has been precipitated at its isoelectric point and washed with distilled water according to the method of Baker and Van Slyke, only enough sodium hydroxide was added to bring the casein to a neutral reaction. In this our method differed from that of Hammarsten (48) in which enough sodium hydroxide is added to dissolve completely the casein.

Loeb's observation suggests that the casein that persists at a neutral reaction is largely combined with divalent bases. We have accordingly removed and discarded this precipitate, either by centrifugation (in a Sharpless centrifuge at nearly 30,000 revolutions a minute) or by filtration through filter paper pulp. The casein in the filtrate or in the centrifugate was then reprecipitated at its isoelectric point by the addition of hydrochloric acid, washed, and again dissolved by sodium hydroxide. It has been our experience that the casein completely dissolved the second time at a neutral reaction. The casein in this clear neutral solution was finally precipitated and purified at the isoelectric point in the manner that has already been described.

Casein has generally been supposed to be nearly completely insoluble. Laqueur and Sackur (49), it is true, observed that a small amount of their casein preparations always dissolved in water, but decided that this amount was negligible. Our experiments upon different preparations show that casein is no less soluble than the globulins that have thus far been prepared in states of comparable purity.

As in the cases of serum globulin and tuberin, the first measurements of the solubility of precipitated casein were high. In this case also this can be explained by assuming that casein forms readily soluble compounds. The nature of these compounds and the extent to which they can exist in the neighborhood of the isoelectric point will be considered in another communication. The solubility of two preparations of uncombined casein has been determined. The results of thirty-two nitrogen determinations made upon the casein dissolved in fivedifferent flasks, containing different amounts of precipitate, on 3 different days, agree within the errors of measurement. Since the earlier work, though not nearly so accurate, is in fair agreement with these later determinations, the average may be taken as the most probable solubility of casein, and yields the result that 0.11 gm. of casein dissolves in 1 liter of water at 25°C.

Many of the measurements that are reported were made by Miss Jessie L. Hendry. It gives me pleasure to express my indebtedness for her invaluable aid.

TABLE VI.

Solubility of Casein III.

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H_2O at beginning of experiment.	
		mg. 4.0	mg. 20.0
		Protein nitrogen in 25 cc. filtrate.	
	1921	mg.	mg.
33	Nov. 29	0.36	
	Dec. 6	0.38	0.35
	1922		
	Jan. 31		0.48
	Feb. 5		0.49
37	" 14	0.57	
	" 22	0.57	
	" 24		0.59

TABLE VII.

Solubility of Casein V.

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H_2O at beginning of experiment.				
		mg. 8.55	mg. 17.10	mg. 42.75	mg. 85.50	mg. 171.0
		Protein nitrogen in 25 cc. filtrate.				
	1922	mg.	mg.	mg.	mg.	mg.
39	Apr. 3	0.47	0.42	0.47	0.48	[0.72
		0.42	0.37	0.47	0.50	0.92
		0.42	0.47	0.50	0.47	0.72]
	" 11	0.50	0.40	0.50	0.45	0.50
		0.35	0.36	0.45	0.45	0.51
		0.40	0.55	0.45		0.50
	" 14			0.40	0.40	0.40
				0.40	0.50	0.40
				0.40	0.45	0.45
Average.....		0.43	0.43	0.45	0.46	0.46

SUMMARY.

1. Two proteins of the globulin type, serum globulin and tuberin, and the protein of milk, casein, have been purified (*a*) of the other proteins and (*b*) of the inorganic electrolytes with which they exist in nature. The methods that were employed are described.

2. All three proteins were found to be only very slightly soluble in water in the pure uncombined state. The solubility of each was accurately measured at 25.0° ± 0.1°C. The most probable solubility of the pseudoglobulin of serum was found to be 0.07 gm. in 1 liter; of tuberin 0.1 gm. and of casein 0.11 gm. The methods that were employed in their determination are described.

3. Each protein investigated dissolved in water to a constant and characteristic extent when the amount of protein precipitate with which the solution was in heterogeneous equilibrium was varied within wide limits. The solubility of a pure protein is therefore proposed as a fundamental physicochemical constant, which may be used in identifying and in classifying proteins.

4. The concentration of protein dissolved must be the sum of the concentration of the undissociated protein molecule which is in heterogeneous equilibrium with the protein precipitate, and of the concentration of the dissociated protein ions.

5. The dissociated ions of the dissolved protein give a hydrogen ion concentration to water that is also a characteristic of each protein.

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CELL PENETRATION BY ACIDS.

V. Note on the Estimation of Permeability Changes.

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(*Contributions from the Bermuda Biological Station for Research, No. 139, and from the Zoological Laboratory, Rutgers College, New Brunswick.*)

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1. Effects of Tension.

In view of the fact that qualitative studies regarding permeability have sometimes been based upon experiments involving intense muscular activity in the tissue examined, I thought it worth while to measure the effect of stretching upon the rate with which acids penetrate the indicator-containing tissue of the nudibranch *Chromodoris* (*cf.* Crozier, 1916 *a*).

A piece of the mantle fold, about 2 cm. in length by 0.5 cm. in breadth, was freshly secured by cutting from an individual sufficiently large to supply several such pieces. The piece was then adjusted in a holder, sketched in Fig. 1. This was accomplished by tying to either end a short ligature of waxed thread. One of the ligatures was made fast at *a*, while the other, longer, one was passed over the groove *c*. The longer thread carried at its free end a mass which in the different tests was varied from 5 to 50 gm. The tissue, being fixed in this manner, was lowered into the trough, *A*, containing an acid solution. The time required for the internal color change to be brought about was measured with a stop-watch, while the first evidence of pigment loss could be accurately recognized by watching the edge of the tissue through a horizontal microscope. A slip of milk-glass, mounted back of the tissue, increased the precision of the observations.

By employing a strip of the mantle about 2 cm. in length, there was secured an area in the center of the strip which was free from complications due to the presence of tight ligatures at either end. This was checked by experiments in which the tissue, with the threads made fast, was allowed to hang freely without any attached weights.

The crushed regions about the threads were almost instantly changed to a pink color by acids, but the central part gave penetration times agreeing absolutely with those found for control pieces devoid of ligatures. With a little practice, the strip of tissue could be adjusted without introducing any twist. It was found that these strips of mantle tissue could in all cases support, over the period involved in an experiment, a mass of about 50 gm. Beyond 55 gm., tearing effects became evident.

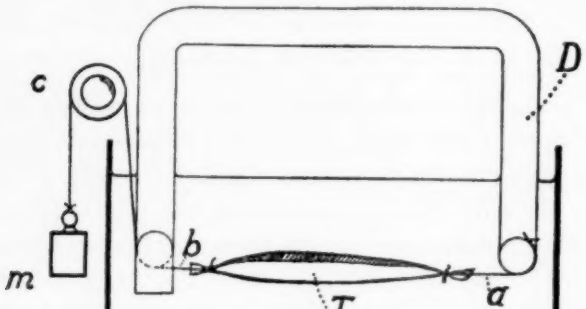

FIG. 1. Side view of device for subjecting to tension, in an acid bath, *A*, a strip of tissue cut from the mantle fold of *Chromodoris*. *T*, tissue; *a*, ligature made fast to glass rod (*D*); *b*, another ligature passing under hook on end of glass rod; and *c*, smooth metal groove (greased), over which ligature from *b* passes to the mass *m*.

The acid chosen for most of the measurements was dichloroacetic, since up to 30 or 40 gm. tension the stronger mineral acids, with higher rates of penetration, give figures which could not always be relied upon as different from those obtained with unstretched tissue. With dichloroacetic, however, the effect of stretching was easily shown.

Experiment 9.26a.—Mantle tissue from a *Chromodoris* 8.5 cm. long; 0.01 N dichloroacetic acid; 25°C.

	min.
(*a*) Control, unstretched, penetration time	6.0
(*b*) With a mass of 10 gm. attached, penetration time	5.5
(*c*) " " " " 20 " " " "	3.7
(*d*) " " " " 40 " " " "	2.8

From a number of tests of this kind, data were secured for the construction of Fig. 2. It is apparent that even moderate tension exerts a very decided effect upon the penetrability of this tissue for 0.01 N dichloroacetic acid. A similar result was had with other weak acids; the coagulative action of the stronger acids tends to obscure the phenomenon.

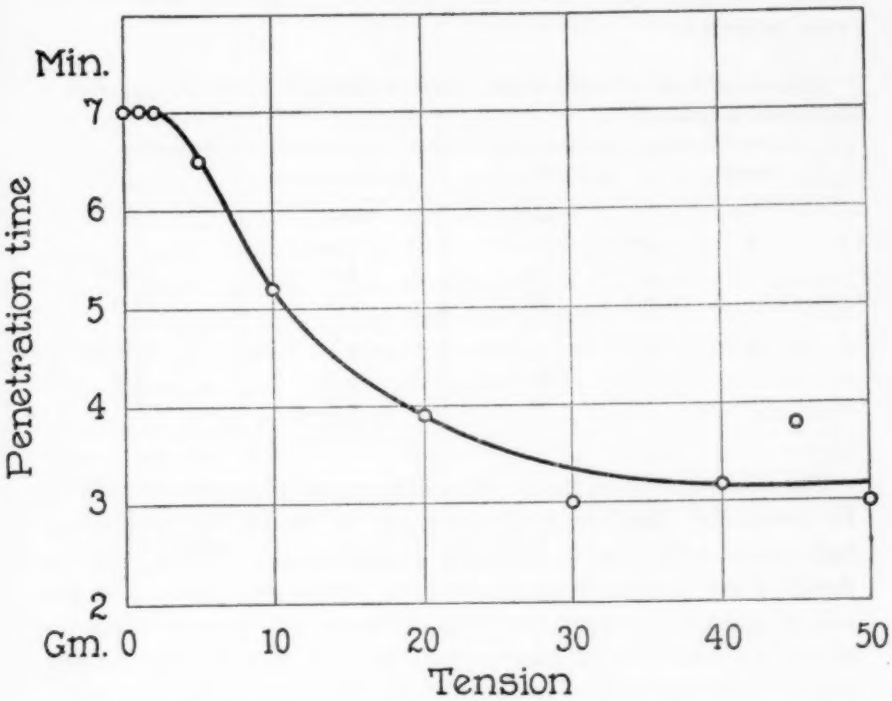

FIG. 2. Penetration of tissues under tension, by 0.01 N dichloroacetic acid; 25°C. Ordinate, penetration time, minutes; abscissa, tension, gm.

Since pieces of mantle tissue subjected for 10 minutes to tensions of 30 to 40 gm., in sea water, quickly regained about their normal penetrability when the traction was removed, the effect of tension is (within limits) reversible.

The effect of tension noted in these experiments cannot be ascribed to manipulation of the tissue during its preparation. Undue

exposure to the air has a very marked influence in the direction of *decreasing* its penetrability. The penetrability of isolated fragments of the *Chromodoris* mantle kept in sea water remained very nearly constant for some 24 hours after their removal.

The influence of tension is equally apparent in relation to diffusion of pigment from the mantle tissue. In one set of experiments, which may be cited as typical, the following observations were recorded:

Experiment 9.26b.—Mantle tissue from two nudibranchs 8.5 cm. long; 0.01 N dichloroacetic acid; 25°C.

Animal.	Stretching mass.	Penetration time.	First sign of pigment loss.
	gm.	*min.*	*min.*
A	0	7.0	15.0
	10	5.5	10.0
	20	3.7	4.0
B	0	7.1	12.0
	30	3.2	4.0
	50	3.0	2.5

The observations on the outward diffusion of pigment are difficult to summarize, because it is necessary to choose for comparison individuals with closely agreeing pigmentations. There was no doubt of the qualitative result, however, which was clearly that the rate of spontaneous pigment diffusion, in acid, is greater when the tissue is stretched. Moreover, the effect of tension was usually greater with respect to pigment diffusion than in relation to penetration of acid (as evidenced by color change). The outcome of these tests may be held to indicate that under tension the resistance of a protoplasmic surface to the diffusion of electrolytes undergoes a (more or less reversible) decrease.[1] The experiments may then have an interesting connection with Carlson's (1907) finding in the

[1] What rôle may be occupied by an actual thinning-out of the cells of the tissue stretched in the present experiments, it is difficult to decide; my chief purpose is to point out, merely, the complication introduced into "permeability" studies by the presence of tractive forces.

Limulus heart, where even moderate traction applied to a cardiac ganglion was seen to increase the intensity of nervous discharge as reflected in the heart beat. And it is obvious that in experiments concerning permeability (*cf.* Lillie, 1909) the presence or absence of relatively intense muscular activity, itself employed as an index of excitation, may introduce a complication difficult to evaluate.

2. *Effects of Stimulation.*

Pieces of the mantle of the nudibranch were cut from individuals of medium size. The fragments excised were about 1.5 cm. long. Non-polarizable electrodes, 4 mm. apart, were applied to the pigmented surface. Current was obtained from a Harvard inductorium with two Leclanché cells in parallel in the primary circuit, the secondary coil being at "5." The stimulation was purposely made rather severe. The mantle fragments became contracted when stimulated, since they contained smooth muscle fibres. Considerable quantities of slimy secretions were expelled from the stimulated surface. After being stimulated the piece of tissue was immersed in an acid solution, and the penetration time was compared with that exhibited for a control piece previously unstimulated.

Experiment 8.33.2.—Four pieces of tissue, each stimulated for 30 seconds, and four control pieces, unstimulated; 0.10 N HNO_3; 21°C.

	Penetration time.	
	Unstimulated.	Stimulated.
	min.	*min.*
(*a*)	6.5	3.2
(*b*)	6.0	2.5
(*c*)	6.0	4.0
(*d*)	6.5	5.0
Mean.	6.3	3.7

The pronounced increase of penetrability demonstrated in such tests was found to hold for other acids as well.

The increase of penetrability is somewhat augmented according to the duration of the stimulation.

Experiment 8.33.3.—Individual A, 6 cm. long; B, 11 cm. long; 0.05 N HNO_3; 20°C.

Tissue from animal.	Stimulation.	Penetration time.	
		Unstimulated.	Stimulated.
	min.	*min.*	*min.*
A	0.5	2.45	1.5
	0.5	2.5	1.3
	1.0	2.5	0.9
B	0.5	5.5	5.5
	1.0	5.5	3.7
	1.0	5.5	3.0
	1.5	5.6	3.7

This effect can hardly be referred to the contraction of the tissue; the mantle fragments relaxed when the electrodes were removed, before being placed in acid; and they always contracted upon immersion in acid, whether previously stimulated or not. Nor can the extruded mucus be directly involved, for the same reason. Moreover, as pointed out beyond, mucus extrusion also occurs under the influence of chloroform, which has an opposite effect upon penetrability.

When a small spot upon a fair sized piece of the nudibranch integument was stimulated, and the whole then placed in acid, the increased penetrability was manifest merely upon the immediate site of excitation. Thus, in one experiment with 0.05 N HNO_3, a stimulated area turned pink in 2.45 minutes, while the general surface of the tissue did not do so until 3.20 minutes had passed, in spite of the fact that mucus discharge had occurred over the entire surface.

The method of observation is such that the results may not be interpreted in terms of "permeability," since the intracellular color change serving as an index of acid penetration is dependent upon the relation of the pigment to the rest of the cell contents; this relation might be altered by excitation of the epithelium. Nevertheless, it is entirely probable that in these tests the penetrability of the protoplasm toward acid is markedly increased as the result of faradic stimulation.

3. *Anesthetics.*

Ether, ethyl alcohol, chloroform, and $MgSO_4$ all produce a very decided increase in the apparent time of penetration of acids.

Experiment 8.54.1.—Tissue from several animals of the same size; acid, 0.05 N HCl. Pieces of mantle immersed for the intervals stated in sea water containing M ethyl alcohol, then transferred to acid.

Tissue from animal.	Time in alcohol solution.	Penetration time.	
		Alcohol.	Control.
	min.	*min.*	*min.*
A	3	5.2	5.0
	3	7.0	5.0
	10	10.0	5.0
	20	10.0	
	20	9.1	
B	2	3.5	2.5
	10	5.0	2.6
	20	9.1	

A number of tests showed essentially similar conditions under the action of other anesthetics. The primary effect of these materials is to decrease the penetrability of the tissue toward acids. Ether, one-half saturated in sea water, and chloroform, one-fifth saturated, decreased the apparent penetration rate of even 0.1 N HCl by 50 per cent, in 2 minutes and in 5 minutes respectively.

4. *Pigment Diffusion.*

The outward diffusion of intracellular coloring matters has frequently been employed as a criterion of increased permeability to the pigment involved, and by inference to other substances also. In addition to other sources of error, this method of observation tends to ignore the fact that the pigment concerned may be held in the cell, not by the state of the cell surface primarily, but by the relation of the pigment, especially when in the form of droplets, to the cytosome as a whole.

As bearing upon the value of pigment diffusion as an index of permeability increase, I would cite experiments showing that the

penetrability of the mantle epithelium of *Chromodoris* toward acids may be caused to vary in the direction either of increase, or of diminution, while the spontaneous diffusion of pigment (uncomplicated by muscular contractions, in this instance,) simultaneously changes in a directly contrary manner. The pigment involved serves as indicator of internal acidity and is at the same time the freely water-soluble coloring matter concerned in visible outward diffusion. For comparative tests it is necessary to use tissues colored in precisely the same way by this pigment.

Experiment 8.34.3.—(*a*) Two pieces of mantle placed in 0.1 N HCl; penetration time (24°)

	min.
	2.16
	2.05

(*b*) Two similar pieces placed in sea water one-half saturated with $CHCl_3$, for 5 minutes; then put into 0.1 N HCl; penetration time (24°)

	2.25
	3.75

In (*a*) loss of pigment was evident in 4 and 10 minutes, respectively; in (*b*), within 2 minutes in both cases.

Experiment 8.42.1.—Four pieces of mantle tissue from a single individual were used; two were placed in 0.1 N HNO_3, the penetration times being 3.25 and 3.50 minutes, respectively; two others were placed for 10 minutes in sea water containing 0.1 M urea, then transferred to 0.1 N HNO_3—the penetration times being now 0.5 and 0.3 minutes. In the first case pigment diffusion was observed in 7 minutes, in the second in 9 minutes.

These tests, typical of a number made, show clearly that the condition of the tissue used may differ markedly when regarded from the standpoints (1) of penetrability for acids, and (2) of the readiness with which spontaneous loss of pigment occurs. A long series of observations upon the penetration of various acids showed, in addition, that practically no correspondence obtains between the relative ease of penetration and the speed with which pigment is lost to the surrounding solution. With mineral acids, pigment loss in general followed the internal color change indicative of penetration; while with weak acids, and especially with fatty acids, even in the case of relatively concentrated solutions penetrating with high velocity, loss of pigment usually preceded the internal sign of acid penetration. Quite apart from the fact that it seems inadvisable to speak of "permeability" as a general property, having reference indis-

criminately to all classes of substances, the facts already enumerated make it hazardous to lay much weight upon pigment diffusion as an index of permeability changes (Crozier, 1916 *b*). Thus in Lillie's extensive operations with *Arenicola* larvæ (1913), the fact that in the presence of anesthetics pigment diffusion is retarded, has been urged as evidence for the restraint by anesthetics of permeability-increasing agencies; on the other hand, when mantle tissue of *Chromodoris* is subjected to the action of anesthetics, pigment is lost much more readily than otherwise, even though the resistance of the tissue toward the diffusion of acids is by this action markedly enhanced.

SUMMARY.

The penetration of acid into mantle tissue of *Chromodoris zebra* is accelerated after local faradic stimulation, and is retarded by brief treatment with anesthetic solutions. The spontaneous outward diffusion of intracellular pigment is an inadequate criterion of "permeability." Outward diffusion of pigment and penetration of acid are both facilitated when the tissue is artificially put under tension.

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Carlson, A. J., 1907, On the mechanism of the stimulating action of tension on the heart, *Am. J. Physiol.*, xviii, 149.

Crozier, W. J., 1916, *a*, Cell penetration by acids, *J. Biol. Chem.*, xxiv, 255.

Crozier, W. J., 1916, *b*, On loss of cell pigment as an index of permeability changes, *Science*, xliii, 145.

Lillie, R. S., 1909, On the connection between changes of permeability and stimulation, and on the significance of changes in permeability to carbon dioxide, *Am. J. Physiol.*, xxiv, 14.

Lillie, R. S., 1913, Antagonism between salts and anæsthetics,—III. Further observations showing parallel decrease in the stimulating, permeability increasing, and toxic actions of salt solutions in the presence of anæsthetics, *Am. J. Physiol.*, xxxi, 255.

THE COMBINATION OF GELATIN WITH HYDROCHLORIC ACID.

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(Received for publication, May 12, 1922.)

Titration curves for gelatin with different acids have been obtained by Loeb,[1,2] and used to show that the protein reacts stoichiometrically with the different acids. The amount of a strong acid combined with the protein may be obtained from the titration curve (in which the abscissæ are pH values and the ordinates concentrations of acid) by subtracting from the ordinates the amounts of acid necessary to bring the same volume of water, without protein, to the same pH values. In this way Loeb[2] obtained a curve for the amount of HCl combined with 1 gm. of gelatin at different pH values. Similar curves were obtained by Tague[3] for the combination of amino-acids with NaOH, and by the writer[4] for the combination of edestin with acids. Each of these combination curves appeared to become horizontal beyond a certain pH; *i.e.*, after enough acid (or alkali, in the case of Tague's experiments) had been added, the amount combined with the ampholyte became constant.

In a recent paper by Lloyd and Mayes[5] a curve is given to represent the amount of HCl combined with 1 gm. of gelatin. This curve, however, does not become horizontal, but rises with increasing acidity in a rather discontinuous manner. These authors did not obtain the amount of combined HCl in the way just described, but calculated it by the formula $N' = N - \frac{[H^+]\text{ corr.}}{\alpha}$ where $[H^+]\text{corr.} = \sqrt{[H^+] \times [Cl^-]}$,

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 100.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 50–51.

[3] Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

[4] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

[5] Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc., London, Series B*, 1922, xciii, 69.

N = normality of total HCl, α its degree of ionization, and N′ = normality of combined HCl. The values which they used for the degree of ionization were conductivity ratios given by Lewis.[6] Inasmuch as the results of Lloyd and Mayes differed from those obtained by Loeb, it seemed worth while to repeat and amplify his experiments.

Solutions were made up containing 1, 2.5, and 5 gm. of gelatin in 100 cc. of HCl of various concentrations. The gelatin was taken from a stock solution of isoelectric gelatin which had been rendered practically ash-free in the way described by Loeb,[7] and was diluted to twice the concentration required for each set of experiments. 25 cc. samples of these solutions were measured out at 33°C. by a pipette, and each sample was diluted to 50 cc. by the addition of the proper amounts of 0.1 M or 1.0 M HCl and water from burettes. The concentration of the gelatin was checked by dry weight determinations of the amount of gelatin delivered by the 25 cc. pipette, and was accurate to about 1 part in 200. The concentration of the acid used was determined by titration against pure Na_2CO_3, and was accurate to 1 part in 500 or better. The pH of the solutions was determined at 33° with the hydrogen electrode and potentiometer, using rocking cells of the Clark type connected by a salt bridge of saturated KCl to a saturated KCl calomel cell. The pH values are referred to 0.1000 M HCl as a standard, its pH being taken as 1.036 at 33°. The E.M.F. readings obtained were reproducible to within 1 millivolt, which corresponds to about 0.02 pH.

The titration curves obtained in this way are given in Fig. 1, the abscissæ being pH values, and the ordinates total concentration of HCl expressed in millimoles per liter, which is the same as cc. of 0.1 M acid per 100 cc. The curve for 1 per cent gelatin represents three experiments, one of which was performed by Mr. M. Kunitz.

In order to determine how much of the total HCl was not combined with the gelatin, a series of solutions containing only HCl and water was prepared, and the pH values were determined. The values are given in Table I.

[6] Lewis, W. C. McC., A system of physical chemistry, London and New York, 3rd edition, 1920, ii, 219.

[7] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39; *J. Am. Chem. Soc.*, 1922, xliv, 213; Proteins and the theory of colloidal behavior, New York and London, 1922, 35.

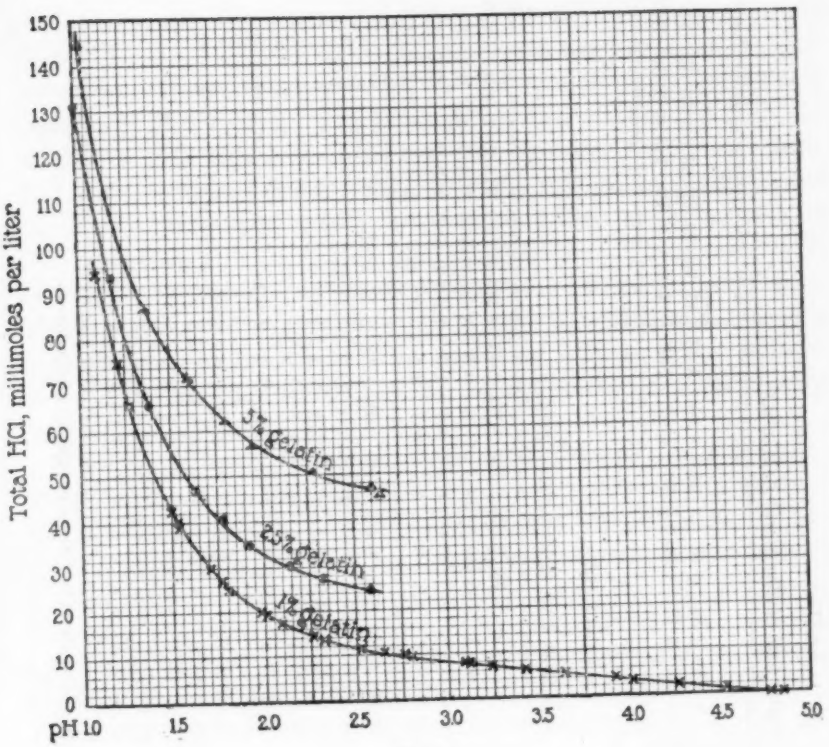
Total HCl, millimoles per liter
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
pH 1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5% Gelatin
2.5% Gelatin
1% Gelatin

FIG. 1.

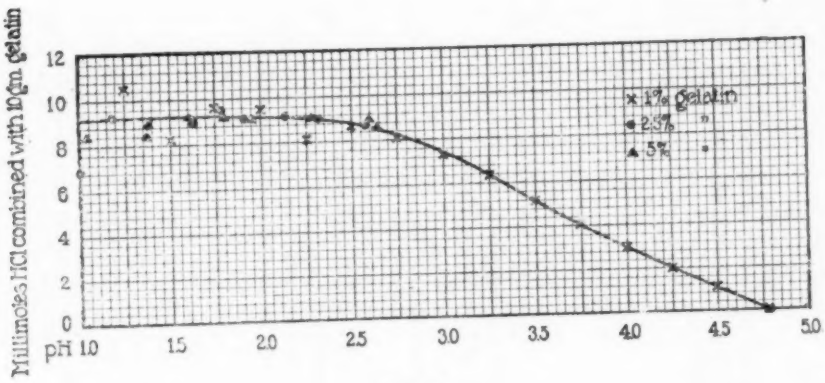
Millimoles HCl combined with 1 gm. gelatin
12
10
8
6
4
2
0
pH 1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
× 1% Gelatin
• 2.5% "
▲ 5% "

FIG. 2.

TABLE I.

Titration of Water with Hydrochloric Acid at 33°C.

Concentration of HCl, per liter, *millimoles*	0.05	0.10	0.19	0.39	0.68	0.97	1.95	3.89	8.75	15.56	24.31	48.63	100.00	117.7
pH	4.35	4.07	3.74	3.38	3.17	3.00	2.70	2.41	2.06	1.81	1.63	1.33	1.04	0.99

TABLE II.

Titration of Gelatin with Hydrochloric Acid.

pH of 1 per cent gelatin chloride*	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50
Total HCl per liter, *millimoles*	69.5	41.3	27.6	19.5	14.7	11.8	9.8	8.3	6.8	5.5	4.3	3.1	2.0	1.1
Uncombined HCl	59.0	33.1	18.0	10.0	6.6	3.1	1.7	1.0	0.5	0.3	0.2	0.1	0.0	0.0
Combined HCl per 10 gm. gelatin, *millimoles*	10.5	8.2	9.6	9.5	8.1	8.7	8.1	7.3	6.3	5.2	4.1	3.0	2.0	1.1
pH of 2.5 per cent gelatin chloride	1.00	1.18	1.38	1.63	1.78	1.91	2.14	2.32	2.58					
Total HCl per liter, *millimoles*	131.3	93.8	65.7	46.9	40.8	35.0	30.2	27.2	24.3					
Uncombined HCl	114.0	70.5	43.5	24.3	16.7	12.3	7.3	4.8	2.6					
Combined HCl per 25 gm. gelatin, *millimoles*	17.3	23.3	22.2	22.6	24.1	22.7	22.9	22.4	21.7					
Combined HCl per 10 gm. gelatin, *millimoles*	6.9	9.3	8.9	9.0	9.5	9.1	9.2	9.0	8.7					
pH of 5 per cent gelatin chloride	1.03	1.37	1.61	1.80	1.95	2.28	2.60	2.64						
Total HCl per liter, *millimoles*	145.0	86.6	71.5	62.1	56.5	50.8	47.1	45.2						
Uncombined HCl	103.0	44.4	25.5	15.9	11.2	5.3	2.5	2.3						
Combined HCl per 50 gm. gelatin, *millimoles*	42.0	42.2	46.0	46.2	45.3	45.5	44.6	42.9						
Combined HCl per 10 gm. gelatin, *millimoles*	8.4	8.4	9.2	9.2	9.1	9.1	8.9	8.6						

* The figures for 1 per cent gelatin chloride were obtained from the curve averaging three experiments.

These results were plotted on a large scale and a smooth curve was drawn through the points. From this curve were read off the concentrations of free HCl present at the pH actually measured in each gelatin chloride solution, and these values were subtracted from the total HCl concentrations of the respective gelatin chloride solutions. The differences accordingly represent the millimoles of HCl combined with the amount of gelatin present in 1 liter, or the cc. of 0.1 M HCl combined with the gelatin present in 100 cc. These values were divided by the number of grams of gelatin in 100 cc. to get the cc. of 0.1 M HCl combined with 1 gm. of gelatin, which is the same as the number of millimoles of HCl combined with 10 gm. of gelatin. Table II indicates the method of calculation, and the final results are plotted in Fig. 2.

It will be noticed that the points lie fairly close to a smooth curve, except in the most acid region, where a small error in the pH may lead to a large error in the difference between the ordinates of two steep curves. The curve is horizontal between pH 1 and 2, indicating that here the gelatin is all combined with the acid. There is no evidence of the discontinuous sections found in the curve of Lloyd and Mayes.[5] This difference is due in part to differences in the experimental curves, but is also due largely to the method of calculation of the combined acid. The method used by Lloyd and Mayes involves the assumption that the uncombined HCl is ionized to the extent indicated by the conductivity ratio for a different concentration of HCl; namely, the concentration of the total HCl present. Moreover, these authors have neglected the difference between the conductivity ratios and the activity coefficients or hydrogen electrode values for HCl, which is clearly brought out by the table given by Lewis[6] from which they obtained their ionization values. The method of calculation used by Loeb, Tague, and the present writer involves the assumption that the same concentration of uncombined HCl is necessary to furnish the same hydrogen ion concentration, as determined by the hydrogen electrode, whether or not gelatin is present. The latter assumption seems to lead to more reasonable results.

The maximum height of the curve in Fig. 2, 9.2 millimoles of HCl for 10 gm. of gelatin, indicates that a 1 per cent gelatin solution has a

normality of 0.0092 with respect to its combination with HCl, or that the combining weight of gelatin is $\frac{10}{0.0092}$, or about 1,090. While the exact height of the maximum is still more or less uncertain, it is probable that this value of the combining weight is more nearly correct than the smaller values given by Procter,[8] Wilson,[9] Wintgen and Krüger,[10] and Wintgen and Vogel,[11] because the calculation involves simpler and more probable assumptions. Moreover, the earlier workers did not have ash-free or isoelectric gelatin at their disposal.

It is possible to calculate from these results an ionization constant for gelatin, assuming it to react as a mono-acid base. The simplest way of doing this is based on the resemblance between the combination curve and the dissociation curve of a base. The equation for the ionization of such a base is $\alpha = \frac{k_b}{k_b + [OH^-]}$ where α represents the fraction ionized and k_b the ionization constant. Since the ion product of water, k_w, is equal to $[H^+] \times [OH^-]$, this becomes

$$\alpha = \frac{[H^+]}{[H^+] + K}, \text{ if } K = \frac{k_w}{k_b}$$

Michaelis[12] has pointed out that if α is plotted against log $[H^+]$, at the point where $\alpha = \frac{1}{2}$, log $[H^+]$ = log K. Applying this to Fig. 2, $\alpha = \frac{1}{2}$ where the ordinate = 4.6 or pH = pK = 3.625. Accordingly $K = 2.4 \times 10^{-4}$. This is of the same order as the value obtained by Procter and Wilson[13] and is intermediate between the values obtained by Wintgen and Krüger[10] and Wintgen and Vogel,[11] but differs by a whole power of 10 from that obtained by Lloyd and Mayes.[5] Inasmuch as the use of this constant leads to only rough agreement with the combination curve in Fig. 2, it is not desired to lay any stress on this calculation. It is quite certain that gelatin is not a mono-acid base, though its combination curve may resemble the ionization curve of such a base.

[8] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313.
[9] Wilson, J. A., *J. Am. Leather Chem. Assn.*, 1917, xii, 108.
[10] Wintgen, R., and Krüger, K., *Kolloid-Z.*, 1921, xxviii, 81.
[11] Wintgen, R., and Vogel, H., *Kolloid-Z.*, 1922, xxx, 45.
[12] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 20.
[13] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

SUMMARY.

The amount of HCl combined with a given weight of gelatin has been determined by hydrogen electrode measurements in 1 per cent, 2.5 per cent, and 5 per cent solutions of gelatin in HCl of various concentrations, by correcting for the amount of HCl necessary to give the same pH to an equal volume of water without protein. The curve so obtained indicates that the amount of HCl combined with 1 gm. of gelatin is constant between pH 1 and 2, being about 0.00092 moles.

The writer wishes to express his gratitude for the advice of Dr. Jacques Loeb, under whose direction this work was done.

THE MECHANISM BY WHICH TRIVALENT AND TETRAVALENT IONS PRODUCE AN ELECTRICAL CHARGE ON ISOELECTRIC PROTEIN.

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I.

Experiments on anomalous osmosis recently published[1] suggest that isolectric gelatin assumes a negative charge when a solution of a salt with tetravalent anion, *e.g.* $Na_4Fe(CN)_6$, is added; and that isoelectric gelatin assumes a positive charge when a salt with a trivalent cation, *e.g.* $LaCl_3$, is added. In these experiments special care was taken that the pH of the solutions was that of the isoelectric point of gelatin; *i.e.*, pH 4.7. $CaCl_2$, Na_2SO_4, and NaCl had apparently no such effect on the charge of isoelectric gelatin.

The question arose whether it was possible to support this suggestion by direct measurements of the P.D. between isoelectric solid gelatin and the surrounding salt solution of pH 4.7; and if this was the case what was the reason for this peculiar action of trivalent and tetravalent ions.

The method of the experiments was as follows: Doses of 1 gm. of powdered gelatin (going through mesh 30 but not through 60) were rendered isoelectric in the way described in previous publications. They were then put for 2 hours at 15°C. into 200 cc. of solutions of $LaCl_3$ of different concentration, all of pH 4.7. In this time equilibrium was practically established between the gel and the outside $LaCl_3$ solution. The suspension was then transferred into cylinders and the gelatin granules allowed to settle (at 15°C.). The supernatant liquid and the solid gelatin granules were then separated by filtration, the solid gelatin granules were melted by warming, poured into cylinders with two glass tubes attached (as described in

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

a recent book[2]) and the gelatin was allowed to solidify at 15°C. The P.D. between the solid gelatin and the supernatant $LaCl_3$ solution was then measured with two identical saturated KCl-calomel electrodes connected with a Compton electrometer as previously described.[2] Table I gives the observed P.D. The $LaCl_3$ solutions used varied between M/24 and M/98,304.

The gelatin was nearly but not entirely isoelectric, since it had a positive charge of about 15 millivolts. This means that its pH was not 4.7 but probably about 4.6 or possibly slightly less.

Table I shows that the P.D. rises upon the addition of increasing quantities of $LaCl_3$ from 15 to 23.5 millivolts at a concentration of M/6,144 $LaCl_3$. When the concentration of $LaCl_3$ rises beyond this point, the P.D. decreases again until it becomes 5.0 at a concentration of M/24 $LaCl_3$. $LaCl_3$ acts, therefore, upon the P.D. of isoelectic gelatin in a similar way to HCl. In the case of HCl it has been proven that a salt is formed between gelatin (or any other isoelectric protein) in which the H ion becomes part of a complex positively charged protein ion while the Cl is the anion. It is natural to assume that the reaction is similar to that between NH_3 and HCl where the salt NH_4Cl is formed, inasmuch as the protein contains NH_2 or NH groups in which the N is still able to attract and hold electrostatically another H ion. The correctness of this view is supported by the fact that when we add HCl to a 1 per cent solution of gelatin and measure the Cl potential of the solution, the Cl potential is the same as when we add the same amount of HCl to the same quantity of water (without gelatin), while the H potential is considerably lower in the gelatin solution than in the pure aqueous solution.

When we add increasing concentrations of HCl to solid isoelectric gelatin, gelatin chloride is formed and the ionization of the protein leads to the establishment of a Donnan equilibrium between the solid gelatin and the surrounding aqueous solution, and the unequal distribution of the oppositely charged ions inside and outside the gel is responsible for the P.D.[3] The theory of membrane equilibria

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 154.

[3] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120 ff; *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351, 463.

demands that the P.D. increase with the increase in the concentration of gelatin ions formed, *i.e.* with the increase in the concentration of the acid added, and that the P.D. diminish with the increase of the concentration of Cl ions. At first, the augmenting influence of the acid (through formation of gelatin chloride) increases more rapidly with increasing concentration of acid than the depressing effect of the Cl ions, until a certain part of the gelatin is transformed into ions. From that point on the increase in P.D. due to the ionization of the gelatin increases less rapidly than the depressing effect of the Cl ions and the P.D. diminishes again when more acid is added. This has all been discussed more fully and need not be repeated here.[3]

The experiments mentioned in Table I show that $LaCl_3$ acts in a way entirely similar to HCl. When $LaCl_3$ solutions of low concentration (and of pH 4.7) are added to isoelectric gelatin, the P.D. rises with increasing concentration of $LaCl_3$ until the concentration is M/6,144, when the P.D. falls again. This suggests that the La ion of the $LaCl_3$ solution enters into combination with gelatin, as does the H in the case of HCl, by giving rise to gelatin-lanthanum chloride, in which the cation is a complex positively charged gelatin-La ion, while the anion is the Cl ion. The increase in ionization of the gelatin causes an increase in the P.D., while the Cl ion depresses the P.D. just as if HCl had been added. There is this difference between the gelatin chloride formed by the reaction between isoelectric gelatin and HCl and the gelatin chloride formed by the reaction between gelatin and $LaCl_3$; namely, that the $LaCl_3$ can be easily washed away while the HCl is held more tenaciously.[4] This is easily understood, since the radius of the La ion is so large that in spite of the high charge of the ion it is held with a considerably smaller force by the gelatin than is the H ion, which has no shell of electrons and whose positive nucleus can approach the N of the gelatin very closely.

The action of solutions of $Na_4Fe(CN)_6$ of pH 4.7 on isoelectric gelatin is similar to that of NaOH. The method was the same as that described in the case of $LaCl_3$. The results are given in Table II, showing that $Na_4Fe(CN)_6$ charges the gelatin negatively, the charge

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 27; *J. Gen. Physiol.*, 1918-19, i, 237.

TABLE I.

P.D. *between Solid Isoelectric Gelatin and Solutions of $LaCl_3$ at pH near (but Probably Slightly Below) 4.7. Solid Gelatin Positively Charged.*

Concentration of $LaCl_3$.	0	M/98,304	M/49,152	M/24,576	M/12,288	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24
P. D., *millivolts* ...	15.0	16.0	20.0	20.0	21.0	23.5	22.5	22.0	17.5	14.0	10.0	9.0	6.0	5.0

TABLE II.

P.D. *between Solid Isoelectric Gelatin and $Na_4Fe(CN)_6$ at pH near 4.7. Solid Gelatin Is Negatively Charged Where Minus Sign Is Added.*

Concentration of $Na_4Fe(CN)_6$.	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
P. D., *millivolts*.....	15.0	−3.0	−9.0	−15.0	−22.0	−13.0	−13.0	−11.0	−8.5	−5.5	−4.0	−2.5	−1.5	−1.0

TABLE III.

Influence of Different Concentrations of NaCl, Na_2SO_4, and $CaCl_2$ on the P.D. *between Solid Gelatin and Outside Solution. Gelatin Always Positively Charged unless Minus Sign Is Added.*

Concentration.	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
NaCl.....	13.0		12.0	9.0	7.0	4.0	2.0	1.0	0	0	0	0	0	0.5	1.0
$CaCl_2$.....			13.0	6.0	9.0	2.5	3.5	3.0	2.0	1.5	1.5	3.0	2.5	2.5	1.0
Na_2SO_4.....		7.0	4.5	0.5	−0.5	−2.0	−2.0	−2.0	−1.5						

increasing with an increase in the concentration of $Na_4Fe(CN)_6$ until the concentration of M/4,096 is reached where the P.D. between solid gelatin and $Na_4Fe(CN)_6$ solution is 22 millivolts, the gelatin having the negative charge. Upon increasing the concentration of $Na_4Fe(CN)_6$ beyond M/4,096 the P.D. diminished again until it became 1 millivolt when the concentration of $Na_4Fe(CN)_6$ was M/8 (Table II).

The next step was to find whether solutions of $CaCl_2$ of pH 4.7 also give a positive charge to isoelectric gelatin and whether solutions of Na_2SO_4 give a negative charge. Table III shows that both of these salts, as well as NaCl, have only a depressing effect on the potential of isoelectric gelatin but cause no increase of P.D. in low concentration.

There is, however, a slight difference between the action of the three salts. While in the case of Na_2SO_4 gelatin becomes slightly negative in concentrations beyond M/512, this does not happen in the case of NaCl and $CaCl_2$. The negative charge is, however, so small that it would not be wise to attribute much importance to this fact. The important fact is that solutions of $LaCl_3$ and $Na_4Fe(CN)_6$ of pH 4.7 have a similar influence on isoelectric gelatin to that of acids and alkalies respectively, while this effect cannot be demonstrated in the case of NaCl, $CaCl_2$, or Na_2SO_4. This would be intelligible on the basis of the assumption that the electrostatic attraction of Na,Cl, SO_4, and Ca ions is not sufficient to cause the formation of any considerable quantity of ionizable gelatin salts when reacting with isoelectric gelatin; at least not in the concentrations of salts used in this experiment. If this surmise is correct, we must consider the fact that the electrostatic attraction of an ion is a function of at least two variables; namely, the number of charges and the radius. If this is taken into consideration it is not to be expected that all divalent ions should be as inactive as Ca or SO_4.

II.

If the influence of $LaCl_3$ and $Na_4Fe(CN)_6$ on the P.D. of solid isoelectric gelatin is due to ionization of the protein and the establishment of a Donnan equilibrium as a consequence of this ionization, this should betray itself also in the osmotic pressure of mixtures of

isoelectric gelatin in solutions of $LaCl_3$ or $Na_4Fe(CN)_6$ of pH 4.7. The osmotic pressure of 1 gm. of isoelectric gelatin in 100 cc. of solution should at first increase with the concentration of $LaCl_3$ or $Na_4Fe(CN)_6$ until a maximum is reached, and the osmotic pressure should then drop again if the concentration of these two salts is increased beyond this point. On the other hand, NaCl, LiCl, $MgCl_2$, $CaCl_2$, or Na_2SO_4 should have either no such effect, or it should be considerably smaller.

A stock solution of concentrated isoelectric gelatin was prepared and so diluted that 1 gm. (by dry weight) of such isoelectric gelatin was contained in 100 cc. of H_2O or various concentrations of the salts mentioned, all of a pH of 4.7. Collodion bags of a volume of about 50 cc. were filled with these gelatin solutions. Each bag was put into a beaker containing 350 cc. of a solution of the same salt and the same concentration as that in which the gelatin was dissolved. These 350 cc. of outside solutions were free from gelatin but had the same pH as the salt solution inside the bag; namely, 4.7. The osmotic pressure was measured in the way previously described and the final measurements were made after 18 or 20 hours. The temperature was kept constant, in some cases at 24°, but generally at 27°C. The higher temperature was chosen to prevent the gelatin from setting to a gel too quickly; *i.e.*, before osmotic equilibrium between the gelatin solution and the outside solution was established. Isoelectric gelatin is rather insoluble but becomes more soluble if salt is added. We shall discuss this more fully in a later part of this paper.

The results of these experiments are given in Table IV. From this table it is clear that the addition of $LaCl_3$ and $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ acts on the osmotic pressure of 1 per cent solutions of isoelectric gelatin in a similar way as the addition of acid or alkali, inasmuch as the addition of little $LaCl_3$ or $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ raises the osmotic pressure until a maximum is reached at a concentration of M/2,048 for $LaCl_3$ and of M/4,096 for $Na_4Fe(CN)_6$; while the addition of more $LaCl_3$ or $Ce(NO_3)_3$ or $Na_4Fe(CN)_6$ depresses the osmotic pressure. None of the other salts used, NaCl, LiCl, $MgCl_2$, $CaCl_2$, or Na_2SO_4, acted in this way. There was possibly a slight rise when the concentration of these latter salts became very high.

These experiments confirm the conclusion reached in connection with the experiments on the charge of solid gelatin that salts with trivalent cations or tetravalent anions ionize isoelectric gelatin and thereby set up a Donnan equilibrium; while salts of the type of $CaCl_2$ or Na_2SO_4 or NaCl do not act this way.

In the experiments just described it seemed advisable to measure also the influence of different salt solutions of pH 4.7 on the P.D. between solutions of isoelectric gelatin inside the collodion bag and the outside aqueous solution free from gelatin but also of pH 4.7. These measurements were taken at the end of the experiments after

TABLE IV.

Influence of Salt Solutions of pH 4.7 on the Osmotic Pressure (in Mm. H_2O) of 1 Per Cent Solutions of Isoelectric Gelatin.

Concentration.	0	M/65,536	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
$Na_4Fe(CN)_6$	18			51	94	95	90	75	63	51	48	41	43	38					
Na_2SO_4	30						27	25	30	29	33	32	26	32	33	29			
NaCl	23	22	22	23	23	22	23	27	28	31	25	29	26	28	28	30	35	38	
LiCl							26	26	28	27	30	28	32	36	38	39	40	43	
$MgCl_2$		23	20	24	27	23	28	24	28	28	27	29	33	31	33	36	40	43	42
$CaCl_2$		22	25	24	26	28	26	30	32	29	27	32	30	31	33	40	38	38	40
$LaCl_3$	27	26	32	40	51	52	60	62	57	57	54	50	48						
$Ce(NO_3)_3$	23	28	34	46	50	58	71	69	63	55	50	45							

osmotic equilibrium was established. The figures show that the addition of little $LaCl_3$ or $Ce(NO_3)_3$ increases the P.D. across the membrane until a maximum is reached at a concentration near M/8,192 or M/4,096 and that the P.D. drops again with a further increase in the concentration of the salt. The gelatin is positively charged, showing that the gelatin forms cations. $Na_4Fe(CN)_6$ acts similarly except that gelatin is negatively charged. NaCl, LiCl, Na_2SO_4, $MgCl_2$, and $CaCl_2$ create no potential difference; or in other words, they cause no ionization of isoelectric gelatin. These results agree with the results obtained with solid gelatin and they agree also with the results obtained in the experiments on anomalous osmosis (Table V).

TABLE V.

Influence of Salt Solutions of pH 4.7 on Membrane Potentials in Millivolts between Solutions of Isoelectric Gelatin and Aqueous Solutions Free from Gelatin. Gelatin Solution Positive unless Minus Sign Is Added.

Concentration.	0	M/65,536	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
$Na_4Fe(CN)_6$	−3.0			−9.0	−13.5	−10.0	−6.0	−4.0	−3.0	−1.5	0	0	−0.5	−0.5					
Na_2SO_4	−1.0						−0.5	0	−0.5	0	0	0	0	0	0	0	0		
NaCl	1.0			3.0	2.0	0.5	1.0	0.5	0	0	−0.5	0	0	−0.5	0	0	0	0	
LiCl	0.5						0	−1.0	−0.5	0	0	0	0	−0.5	0	0	0	0	
$MgCl_2$				0	0	0	0	0	0	0	0	0	0	0	−0.5	−0.5	−1.0	−1.5	0
$CaCl_2$	−1.0			0.5	1.0	1.5	1.5	0.5	0.5	0.5	0	0	0	0	0	−0.5	−0.5	−0.5	−1.0
$LaCl_3$	−0.5	1.0	3.0	5.0	8.0	5.0	4.0	3.5	3.0	2.0	1.5	1.0	1.0						
$Ce(NO_3)_3$	−2.5	1.0	5.0	6.5	6.0	7.5	7.0	5.0	3.5	2.0	1.0	0.5							

TABLE VI.

Influence of Solutions of $LaCl_3$ on pH of 1 Per Cent Solutions of Nearly Isoelectric Gelatin.

Concentration of $LaCl_3$	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64
pH	4.85	4.80	4.78	4.70	4.64	4.57	4.52	4.45	4.39	4.34

III.

While all this seems clear, there remains the possibility that the effects of $LaCl_3$ and of $Na_4Fe(CN)_6$ on the P.D. and osmotic pressure of isoelectric gelatin are due to a different kind of ionization from that assumed above. When a solution of $LaCl_3$ of pH 4.7 is added to a gelatin solution of the same pH, the solution becomes more acid; and when a solution of $Na_4Fe(CN)_6$ of pH 4.7 is added to a solution of isoelectric gelatin, the solution becomes more alkaline. A solution of $LaCl_3$ of pH 4.7 makes, therefore, isoelectric gelatin more acid and this should lead to a formation of gelatin chloride, without the necessity of assuming the formation of a complex gelatin-La cation; while solutions of $Na_4Fe(CN)_6$ of pH 4.7 make isoelectric gelatin more alkaline and this should lead to a formation of Na gelatinate. To test this possibility 1 per cent solutions of nearly isoelectric gelatin (pH 4.85) were made up in various concentrations of $LaCl_3$ of pH 4.7 and the pH was determined electrometrically (Table VI).

According to Table V the highest P.D. is reached in $LaCl_3$-gelatin mixtures at a concentration of $LaCl_3$ of M/8,192; such a solution influences the pH of the gelatin solution too little to account for the P.D. of 8.0 millivolts, since near the isoelectric point the difference between pH inside and outside is almost zero.

It was then attempted to repeat the experiments on the influence of $Ce(NO_3)_3$ on the P.D. and osmotic pressure of solutions of isoelectric gelatin in buffer solutions, but these experiments failed since the addition of the salt contained in the buffer solution in itself sufficed to suppress the P.D. to be expected.

To reach a decision the following experiments were made.

1 per cent solutions of isoelectric gelatin were prepared and to some solutions was added HCl to others NaOH so that the pH values obtained were approximately 3.5, 4.0, 4.4, 4.7, 5.0, and 5.5. These gelatin solutions were made up in solutions of different salts of the same pH. In this way it was possible to ascertain the influence of these salts on the pH of the gelatin solution (Table VII).

The P.D. between these gelatin solutions inside the collodion bags and the outside aqueous solutions of the same salt and originally

of the same pH as the gelatin solution (but without gelatin), was also measured after osmotic equilibrium was established. The figures for the P.D. are always under the figures for the pH of the gelatin-salt solutions. The result is very striking. The gelatin is always positively charged when the gelatin is dissolved in M/768 $LaCl_3$ regardless of the pH. Even when the pH of the solution is on the alkaline side of the isoelectric point the gelatin is positively charged in the presence of M/768 $LaCl_3$. No such result is observed when the salt added is NaCl, $CaCl_2$, or Na_2SO_4. In this case the P.D. across the membrane was zero at pH 4.7 and 4.8 and the gelatin became negative when the pH became 5.0 or above. This shows that the positive charge of isoelectric gelatin in the presence of

TABLE VII.

		Gelatin-acid salts.			Isoelectric gelatin.	Metal gelatinates.	
M/768 $LaCl_3$.	pH	3.66	3.98	4.32	4.72	4.82	5.3
	P.D., *millivolts*	+8.5	+7.0	+5.5	+3.5	+3.0	+2.0
M/512 $CaCl_2$.	pH	3.62	4.02	4.44	4.72	5.02	5.43
	P.D., *millivolts*	+8.5	+5.5	+2.5	0	−1.0	−2.5
M/256 NaCl.	pH	3.66	3.98	4.49	4.80	5.19	5.68
	P.D., *millivolts*	+9.5	+7.0	+2.5	0	−3.0	−6.0
M/512 Na_2SO_4.	pH	3.63	4.06	4.41	4.83	5.16	5.82
	P.D., *millivolts*	+6.0	+4.0	+1.0	0	−2.0	−3.0

$LaCl_3$ of pH 4.7 cannot be due to the change in pH but must be due to some other cause of ionization of gelatin such as the formation of a complex gelatin-La cation.

The pH for $Na_4Fe(CN)_6$ could not be measured electrometrically.

In view of all these results we must draw the conclusion that salts with trivalent (and probably also tetravalent) cations cause isoelectric gelatin and gelatin on the alkaline side of the isoelectric point (but near this point) to assume a positive charge owing to the formation of positively charged protein ions, probably of the type gelatin-La. This explains the reversal of the charge of gelatin by trivalent cations observed in anomalous osmosis and kindred phenomena. Tetravalent anions confer a negative charge on isoelectric gelatin and the mechanism is probably similar. Changes of

hydrogen ion concentration may enter to some extent into these effects but they are probably of only secondary importance. No such effects have thus far been obtained with salts possessing divalent or monovalent ions; *i.e.*, salts of the type Na_2SO_4, $CaCl_2$, and NaCl.

IV.

In a gelatin chloride solution of pH 3.0, the greater part of the gelatin is ionized, and since the H ion is held more firmly by the gelatin than the La ion, the addition of $LaCl_3$ to a solution of gelatin chloride of pH 3.0 should not have any augmenting effect on the P.D. or the osmotic pressure of the gelatin chloride solution. The only effect the addition of $LaCl_3$ to a solution of gelatin chloride should have is the depressing effect of the Cl ions. Hence when we mix a solution of gelatin chloride of pH 3.0 with solutions of $LaCl_3$ of the same pH the effect on the P.D. and the osmotic pressure should be merely a depression of the values of these latter properties, and the depression should be quantitatively identical with the effect of $CaCl_2$ or NaCl solutions of the same concentration of Cl ions and the same pH.

This fact had already been ascertained in previous experiments already published,[3] but since these direct measurements of P.D. are of such importance for the theory of the origin of the P.D. between colloids and aqueous solutions it seemed advisable to repeat them. 1 gm. of gelatin chloride of pH 3.0 was dissolved in 100 cc. of solutions of various salts all brought to pH 3.0 through the addition of HCl. Collodion bags were filled with these solutions of gelatin chloride in different salts and each bag was dipped into 350 cc. of an aqueous solution of the same concentration of the same salt and the same pH as that inside the collodion bag, but without gelatin. Water diffused into the collodion bag until osmotic equilibrium was established, and the next day the final osmotic pressure and the P.D. between gelatin solution and outside aqueous solution were measured. The solutions of the three salts were prepared in such a way as to have the same concentration of Cl. In Fig. 1 are plotted the P.D. as ordinates over the concentration of the Cl ions as abscissæ. It is obvious that the influence of NaCl, $CaCl_2$, and $LaCl_3$

on the P.D. is identical for the same concentration of Cl. This proves that only the Cl ion of these salts influences the P.D. of a gelatin chloride solution of pH 3.0 and that the La ion does not increase the P.D. of the solution.

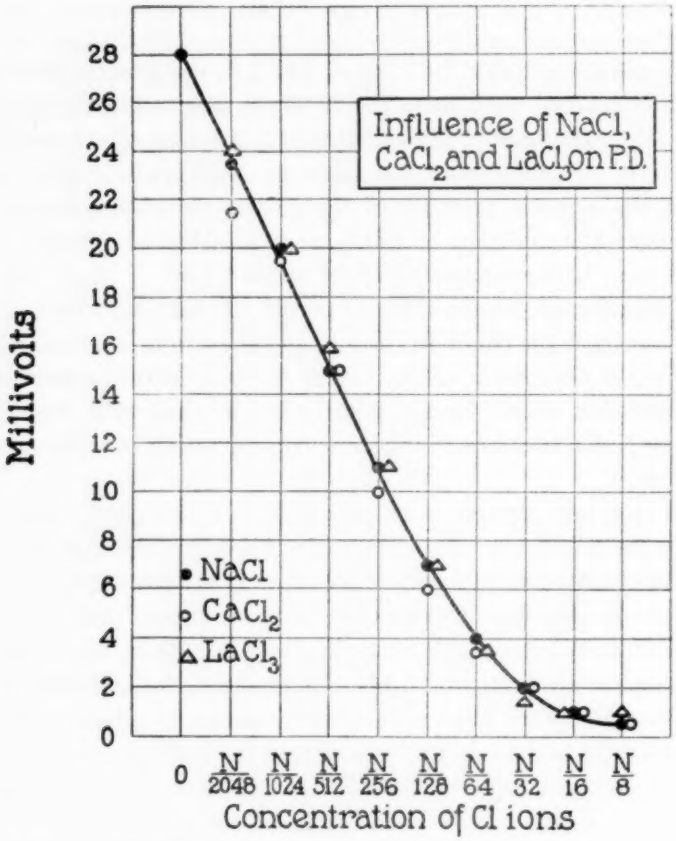

FIG. 1. Depressing influence of NaCl, $CaCl_2$, and $LaCl_3$ on the P.D. between a 1 per cent solution of gelatin chloride of pH about 3.0 and aqueous solutions of the salts originally of the same pH, the two solutions being separated by collodion membranes. Ordinates are the P.D., abscissæ the concentrations of Cl ions of the salts. The depressing effect of the three salts is the same for the same concentration of Cl ions, proving that the cations do not influence the P.D. of gelatin chloride solutions.

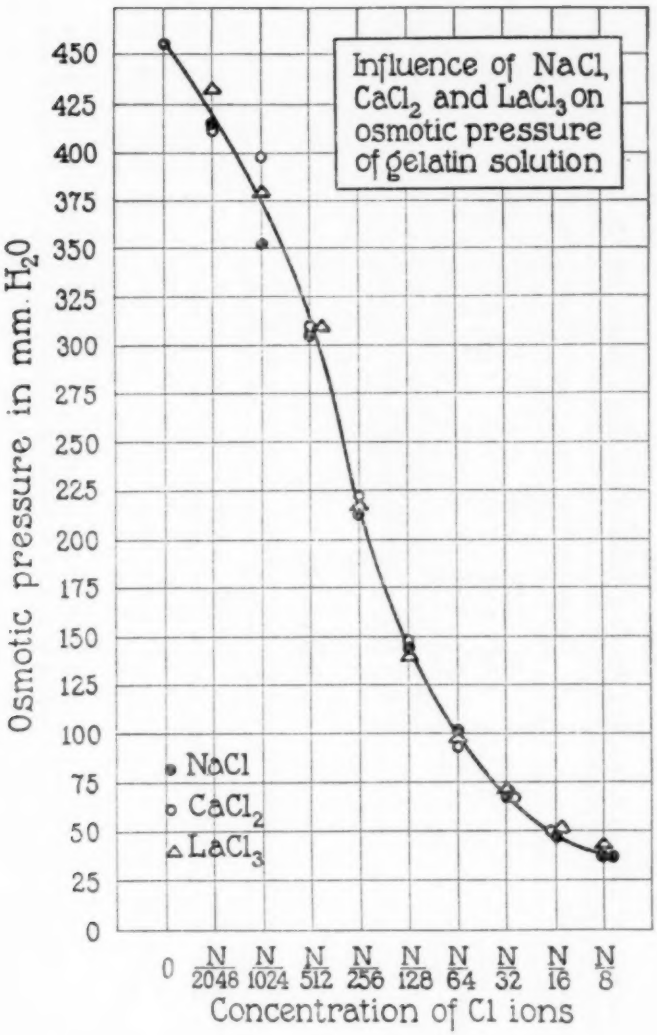

FIG. 2. Influence of NaCl, $CaCl_2$, and $LaCl_3$ on osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.0. Ordinates are osmotic pressure in mm. H_2O, abscissæ the concentrations of Cl ions of the salts. The depressing effect is the same for the three salts, proving that only the anion influences the osmotic pressure in this case.

TABLE VIII.

Influence of NaCl on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of NaCl.	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside	3.35	3.29	3.25	3.21	3.15	3.09	3.06	3.05	3.05	3.06
pH outside	2.88	2.89	2.90	2.93	2.95	2.96	2.98	3.01	3.02	3.05
pH inside minus pH outside	0.47	0.40	0.35	0.28	0.20	0.13	0.08	0.04	0.03	0.01
P.D. calculated, *millivolts*	+28.0	+23.5	+20.5	+16.5	+12.0	+7.5	+4.5	+2.5	+1.8	+0.6
P.D. observed, *millivolts*	+28.0	+23.5	+20.0	+15.0	+11.0	+7.0	+4.0	+2.0	+1.0	+0.5

TABLE IX.

Influence of $CaCl_2$ on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of $CaCl_2$.	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16
pH inside	3.34	3.30	3.24	3.20	3.13	3.09	3.06	3.04	3.04	3.03
pH outside	2.88	2.90	2.91	2.94	2.93	2.96	2.99	2.99	3.01	3.01
pH inside minus pH outside	0.46	0.40	0.33	0.26	0.20	0.13	0.07	0.05	0.03	0.02
P.D. calculated, *millivolts*	+27.0	+23.5	+19.5	+15.5	+11.5	+7.5	+4.0	+3.0	+1.8	+1.2
P.D. observed, *millivolts*	+28.0	+21.5	+19.5	+15.0	+10.0	+6.0	+3.5	+2.0	+1.0	+0.5

TABLE X.

Influence of $LaCl_3$ on the P.D. *of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of $LaCl_3$	0	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24
pH inside	3.33	3.27	3.23	3.18	3.12	3.08	3.06	3.03	3.03	3.02
pH outside	2.88	2.87	2.90	2.93	2.95	2.97	3.01	3.00	3.01	3.01
pH inside minus pH outside	0.45	0.40	0.33	0.25	0.17	0.11	0.05	0.03	0.02	0.01
P.D. calculated, *millivolts*	+27.0	+24.0	+20.0	+15.0	+10.0	+6.5	+3.0	+2.0	+1.0	+0.5
P.D. observed, *millivolts*	+28.0	+24.0	+20.0	+16.0	+11.0	+7.0	+3.5	+1.5	+1.0	+1.0

In these experiments the hydrogen ion concentrations of the inside and outside solutions were measured and the writer begs leave to give these results (Tables VIII, IX, and X), since they prove once more that the P.D. between a solution of gelatin chloride and an outside solution across a collodion membrane is determined by the difference in the pH in the inside and outside solutions, as the Donnan theory demands. In these experiments the influence of the three salts on the osmotic pressure of the gelatin chloride solutions was also measured and the results are given in Fig. 2 showing that the osmotic pressure of a gelatin chloride solution of pH 3.0 is influenced only by the anion but not by the cation of the salt, since the effect of the three salts on the osmotic pressure of the solution is exactly the same when plotted over the concentration of the Cl ions. At this pH, therefore, the La does not increase the osmotic pressure of gelatin chloride solutions.

These observations support the idea that trivalent and tetravalent ions are able to transfer their charge to isoelectric protein by causing the protein to be ionized; probably in such a way that the trivalent or tetravalent ion is part of a complex protein ion.

SUMMARY AND CONCLUSIONS.

1. Experiments on anomalous osmosis suggested that salts with trivalent cations, *e.g.* $LaCl_3$, caused isoelectric gelatin to be positively charged, and salts with tetravalent anions, *e.g.* $Na_4Fe(CN)_6$, caused isoelectric gelatin to be negatively charged. In this paper direct measurements of the P.D. between gels of isoelectric gelatin and an aqueous solution as well as between solutions of isoelectric gelatin in a collodion bag and an aqueous solution are published which show that this suggestion was correct.

2. Experiments on anomalous osmosis suggested that salts like $MgCl_2$, $CaCl_2$, NaCl, LiCl, or Na_2SO_4 produce no charge on isoelectric gelatin and it is shown in this paper that direct measurements of the P.D. support this suggestion.

3. The question arose as to the nature of the mechanism by which trivalent and tetravalent ions cause the charge of isoelectric proteins. It is shown that salts with such ions act on isoelectric gelatin in a way

similar to that in which acids or alkalies act, inasmuch as in low concentrations the positive charge of isoelectric gelatin increases with the concentration of the $LaCl_3$ solution until a maximum is reached at a concentration of $LaCl_3$ of about M/8,000; from then on a further increase in the concentration of $LaCl_3$ diminishes the charge again. It is shown that the same is true for the action of $Na_4Fe(CN)_6$. From this it is inferred that the charge of the isoelectric gelatin under the influence of $LaCl_3$ and $Na_4Fe(CN)_6$ at the isoelectric point is due to an ionization of the isoelectric protein by the trivalent or tetravalent ions.

4. This ionization might be due to a change of the pH of the solution, but experiments are reported which show that in addition to this influence on pH, $LaCl_3$ causes an ionization of the protein in some other way, possibly by the formation of a complex cation, gelatin-La. $Na_4Fe(CN)_6$ might probably cause the formation of a complex anion of the type gelatin-$Fe(CN)_6$. Isoelectric gelatin seems not to form such compounds with Ca, Na, Cl, or SO_4.

5. Solutions of $LaCl_3$ and $Na_4Fe(CN)_6$ influence the osmotic pressure of solutions of isoelectric gelatin in a similar way as they influence the P.D., inasmuch as in lower concentrations they raise the osmotic pressure of the gelatin solution until a maximum is reached at a concentration of about M/2,048 $LaCl_3$ and M/4,096 $Na_4Fe(CN)_6$. A further increase of the concentration of the salt depresses the osmotic pressure again. NaCl, LiCl, $MgCl_2$, $CaCl_2$, and Na_2SO_4 do not act in this way.

6. Solutions of $LaCl_3$ have only a depressing effect on the P.D. and osmotic pressure of gelatin chloride solutions of pH 3.0 and this depressing effect is quantitatively identical with that of solutions of $CaCl_2$ and NaCl of the same concentration of Cl.

IONIZING INFLUENCE OF SALTS WITH TRIVALENT AND TETRAVALENT IONS ON CRYSTALLINE EGG ALBUMIN AT THE ISOELECTRIC POINT.

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I.

INTRODUCTION.

Measurements of the membrane potentials between aqueous protein solutions or gels and surrounding water at equilibrium have yielded the result that salts with trivalent cations give isoelectric protein a positive charge while salts with tetravalent anions give it a negative charge.[1] On the basis of Donnan's theory of membrane potentials it was assumed that salts with trivalent cations, *e.g.* $LaCl_3$, form with isoelectric protein ionizable salts which result in the formation of positive protein-La ions and negative Cl ions; and that salts like $Na_4Fe(CN)_6$ form with isoelectric protein salts which result in the formation of negative protein-Fe $(CN)_6$ ions and positive Na ions.[2] In other words, salts with trivalent cations react with isoelectric protein like acids, and salts with tetravalent anions react with isoelectric protein like alkalies; with this difference, however, that the compounds of isoelectric gelatin with acids and bases are much more stable than those with the salts of trivalent cations or tetravalent anions. Salts with divalent ions like Na_2SO_4, $CaCl_2$, or salts with monovalent ions like NaCl, did not produce any measurable charge on isoelectric gelatin in aqueous solutions. Experiments on anomalous osmosis through gelatin-collodion membranes were in harmony with these results.[3]

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 741.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 165.

[3] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

It seemed of interest to find out whether experiments on the stability of aqueous solutions and suspensions of proteins at the isoelectric point are also in harmony with the results of the direct measurements of the membrane potentials. The reason why experiments on stability and flocculation were selected was that it is often stated that the flocculation of colloids is influenced in an opposite sense by the two oppositely charged ions of a salt, the ion with the same sign of charge as the colloid increasing the stability, the salt ion with the opposite sign of charge to that of the colloidal particle diminishing the stability of the suspension.

If we wish to use observations on the influence of salts on the stability of protein solutions at the isoelectric point for conclusions concerning the influence of ions on the electrical charges of particles, we are confronted with the difficulty that the electrical charges of particles are not the only forces which keep proteins in solution. There are two different kinds of forces determining the stability of solutions or suspensions of proteins, namely; first, the attraction between the molecules of the protein and the solvent, and second, forces of electrostatic repulsion between micellæ. When the forces of attraction between molecules of the solvent and molecules of the solute (which may be secondary valency forces) are greater than the forces of attraction between the molecules of the solute for each other, the solution will be stable. This type of forces acts in the general case of solutions of crystalloids.

When the forces of attraction between the molecules of solute and solvent are weak, the molecules of the solute upon colliding may adhere to each other and aggregates will be formed. This aggregate formation will lead to a flocculation or coagulation of the whole mass unless new forces originate in the small nascent aggregates (or micellæ) which prevent their coalescence into larger aggregates. These forces may be electrical charges whereby the nascent micellæ repel each other. The writer has investigated the origin of these charges in the case of protein micellæ and has found that they are due to the establishment of a Donnan equilibrium between particles and solution.[4] A membrane equilibrium between particles and

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.

solution can, however, only be established when the particles contain protein ions.

There exists a criterion which seems to permit us to decide which one of the two types of forces is responsible for the stability of a solution or suspension. When the stability depends upon the repulsive forces due to a potential difference between micellæ and solution, comparatively low concentrations of solutions will be required for the precipitation of the protein, since comparatively low concentrations of salts (*e.g.* concentrations of M/8 or less) suffice for the annihilation of the P.D. When, however, the stability of a solution is not determined by the P.D. between micellæ and solution but by forces of residual valency between molecules of solute and solvent, much higher concentrations of salts are, as a rule, required for precipitation than are sufficient for the annihilation of a P.D. caused by the Donnan equilibrium. Moreover, the efficiency of a salt in annihilating the P.D. between a micella and a solution is the lower the higher the valency of that ion of the salt which has the opposite sign of charge to that of the micella.

II.

The Prevention of Heat Coagulation of Isoelectric Egg Albumin by Trivalent and Tetravalent Ions.

Isoelectric crystalline egg albumin is quite soluble in water as long as the temperature is low. 8 per cent solutions kept at 2°C. remained perfectly clear for more than a year—and they would probably have kept clear indefinitely. Since it requires very high concentrations of salts to cause a precipitation of isoelectric crystalline egg albumin from aqueous solution at ordinary temperature, we may assume that the forces determining the stability of solutions of isoelectric crystalline egg albumin at sufficiently low temperature are not the electrical charges of micellæ but the attraction between molecules of isoelectric albumin and molecules of water. When, however, the temperature of a 1 per cent solution of isoelectric crystalline egg albumin is raised to about 73°C. or above, crystalline egg albumin is flocculated. Through the rise in temperature a change occurs in the molecule of crystalline egg albumin, whereby the attraction of the molecules of

albumin for each other becomes greater than the attraction between the molecules of albumin and water.

If the albumin is practically non-ionized (as is the case at the isoelectric point) no Donnan equilibrium between the nascent micellæ and the surrounding solution can be established and no P.D. between the nascent micellæ and the solution can prevent the coalescence of the micellæ. When, however, part of the albumin is ionized, the molecules of albumin will also unite upon heating to form micellæ, but these micellæ will begin to repel each other as soon as they contain protein ions. For in this case the protein ions in the nascent micellæ will cause the establishment of a Donnan equilibrium between the micellæ and the solution, and the electrical charge produced thereby on the particles will prevent the further coalescence of the nascent micellæ. This charge will increase with the relative concentration of ionized protein contained in the micellæ. It is obvious that the average size of the micellæ will remain the smaller the greater the relative concentration of protein ions in solution; since the greater the relative concentration of ionized protein the smaller will be the average number of protein molecules which can form an aggregate without including protein ions. This argument is supported by the well known fact that when we add some acid or alkali to isoelectric albumin, the solution will become only opalescent on heating but heat precipitation of the albumin will no longer occur. A comparison of the effect of increasing concentrations of acid shows that the relative size of the micellæ will become the smaller the greater the relative mass of ionized protein. To demonstrate this, 10 cc. of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and containing varying amounts of 0.1 N HCl were put into test-tubes, and these test-tubes were put into boiling water until the temperature of the albumin solution rose to 90°C. Then the test-tubes were allowed to cool at room temperature and the appearance of the solution was noticed. Table I gives the result.

When the 10 cc. contained 0.01 cc. of 0.1 N HCl the protein remained practically isoelectric (pH 4.8), practically no ionization was produced, and hence flocculation occurred upon heating.

TABLE I.

Influence of HCl on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution. 10 cc. of 0.2 Per Cent Albumin (Nearly Isoelectric), Containing Various Concentrations of 0.1 N HCl, Heated to 90°C.

cc. 0.1 N HCl in 10 cc. of 0.2 per cent albumin	0	0.01	0.02	0.03	0.04	0.05	0.1	0.2	0.4	0.8	1.6	3.2
Appearance of solution	Coagulated.		Very opaque.	Very opalescent.	Clear but slightly opalescent.	Very clear, like water.						

The addition of 0.02 cc. of 0.1 N HCl prevented coagulation but the solution was opaque showing that only when the micellæ were comparatively large did they assume electrical charges; owing to the fact that the concentration of albumin ions was small compared with that of non-ionized protein. These charges sufficed, however, to prevent the further coalescence of the large micellæ. When the solutions contained 0.03 cc. of 0.1 N HCl the relative concentration of ionized protein was increased and hence the micellæ remained smaller; the solution was no longer opaque but opalescent. With the addition of 0.04 cc. of 0.1 N HCl the solution became very transparent, showing only slight opalescence. With the increasing concentration of ionized protein the average number of molecules in a micella was considerably diminished, and this small size of the average micellæ manifested itself in the greater transparency of the solution. With a still greater concentration of HCl the average size of the micellæ diminished still further and the solution became as clear as water.

When, however, the concentration of HCl was increased beyond a certain limit, the P.D. between the micellæ and solution was diminished again on account of the depressing effect of the Cl ions demanded by Donnan's theory. When 100 cc. of 1 per cent solution of originally isoelectric albumin contained 30 cc. of N HCl, the protein coagulated at a temperature of 66°C. In this case all the protein was practically ionized but the P.D. between the micellæ and the liquid was nevertheless depressed to zero on acccount of the high concentration of Cl ions.

By measuring the concentration of salt required to precipitate crystalline egg albumin from a 1 per cent solution in water of pH 3.0 at a temperature of 70°C. we can show that the forces preventing heat coagulation in this case are the electrical charges of the micellæ, since the concentration of salt required to cause precipitation is of the order of M/8 or below, and since sulfates are more efficient than chlorides.

The fact that ionization of protein prevents heat coagulation of albumin can be used to find out whether other electrolytes than acids or alkalies are able to produce ionization of isoelectric egg

albumin. If other ions, like La, Ca, Na, SO_4, have such an effect on aqueous solutions of isoelectric albumin, it should show itself in the prevention of heat coagulation and in the optical appearance of the albumin solution after heating.

The experimental procedure was as follows: 7 cc. of water of pH 4.8 (this pH being the isoelectric point of crystalline egg albumin) were added to 2 cc. of 1 per cent solution of isoelectric crystalline egg albumin (of course, also of pH 4.8) and then 1 cc. of a salt solution containing different salts of different concentration, but always of pH 4.8, was added. The test-tubes containing the 10 cc. of the mixtures were put into boiling water until the liquid in the test-tubes reached a temperature of 90°C. and then the test-tubes were taken out of the water bath and allowed to cool at room temperature. Table II gives the appearance of the various mixtures after standing over night.

These experiments show first that the heat coagulation of isoelectric solutions of crystalline egg albumin is prevented by the addition of low concentrations of $LaCl_3$ or $Na_4Fe(CN)_6$ of pH 4.8. The concentration of $LaCl_3$ sufficient for this purpose was M/5,000 and that of $Na_4Fe(CN)_6$ about the same. Hence these two salts acted on the heat coagulation of isoelectric egg albumin like acids or alkali respectively. Moreover, it is obvious from Table II that at first the size of the micellæ formed diminishes with increasing concentration of $LaCl_3$ ions until the molecular concentration of $LaCl_3$ is about M/160. With a further increase of concentration of salt the size of the micellæ increases again (at 3 M/80) owing to the fact that the P.D. is depressed by the Cl ions; and at M/20 $LaCl_3$ this depressing action of the Cl ions on the P.D. is sufficient to permit again the heat coagulation of the albumin. In the case of $Na_4Fe(CN)_6$ the solution ceases to be clear when the concentration becomes 6M/80; in this case the depressing action of the Na ions on the P.D. of the negatively charged micellæ is so great that the micellæ begin to coalesce again.

None of the other salts tried, $CaCl_2$, $BaCl_2$, NaCl, or Na_2SO_4, is able to prevent heat coagulation of isoelectric egg albumin in an aqueous solution of pH 4.8. It is, of course, possible that certain

TABLE II.

Influence of Different Salts on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution at pH of Isoelectric Point.

Total concentration of salt in 10 cc. of 0.2 per cent albumin	10M/80	8M/80	6M/80	4M/80	3M/80	2M/80	M/80	M/160	M/320	M/640	M/1,280	M/2,560	M/5,120	M/10,240	M/20,480	M/40,960	M/81,920	0
$LaCl_3$	Coagulated.				Opalescent.	Bluish clear.							Opalescent and turbid.	Milky.	Coagulated.			
								Clearest.										
$Na_4Fe(CN)_6$		Very opaque.	Slight opalescence.	Clear.						Increasing opalescence. →			Milky.				Coagulated.	
$BaCl_2$			Coagulated.															
$CaCl_2$						Coagulated.												
Na_2SO_4						Coagulated.												
NaCl						Coagulated.												

other bivalent and monovalent ions act differently, since the valency is not the only variable determining the combining action.[5]

These results are in entire agreement with the experiments published in a preceding paper[1] showing that only salts with trivalent and tetravalent ions can produce a membrane potential on isoelectric gelatin while Na, Ca, Ba, and SO_4 have no such effect.

When crystalline egg albumin is dissolved in a solution with little water and much alcohol, salts have not the same influence on the stability of the solution that they have in an aqueous solution. This is due to the difference in the nature of the solvent, since the influence of salts on the stability of such alcoholic solutions of crystalline egg albumin is similar to the influence of salts on the stability of solutions of gelatin in a solution with much alcohol and little water. The stability of isoelectric gelatin in a mixture with little water and much alcohol is increased not only by salts with trivalent and tetravalent ions but also by salts with bivalent ions, such as $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, and Na_2SO_4, while salts like $MgSO_4$, LiCl, NaCl, or KCl have no such effect. The clearing effect of Ba was considerably greater than that of Mg. We know too little about the P.D. and solubility in alcoholic solutions and for this reason the publication and discussion of these results may be postponed.

SUMMARY AND CONCLUSION.

1. While crystalline egg albumin is highly soluble in water at low temperature at the pH of its isoelectric point, it is coagulated by heating. It has long been known that this coagulation can be prevented by adding either acid or alkali, whereby the protein is ionized.

2. It is shown in this paper that salts with trivalent or tetravalent ions, *e.g.* $LaCl_3$ or $Na_4Fe(CN)_6$, are also able to prevent the heat coagulation of albumin at the isoelectric point (*i.e.* pH 4.8), while salts with a divalent ion, *e.g.* $CaCl_2$, $BaCl_2$, Na_2SO_4, or salts like NaCl, have no such effect.

[5] Michaelis, L., and Rona, P., *Biochem Z.*, 1919, xciv. 225.

3. This is in harmony with the fact shown in a preceding paper that salts with trivalent or tetravalent ions can cause the ionization of proteins at its isoelectric point and thus give rise to a membrane potential between micellæ of isoelectric protein and surrounding aqueous solution, while the above mentioned salts with divalent and monovalent ions have apparently no such effect.

ON THE INFLUENCE OF AGGREGATES ON THE MEMBRANE POTENTIALS AND THE OSMOTIC PRESSURE OF PROTEIN SOLUTIONS.

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I.

When a protein chloride solution is separated by a collodion membrane from a dilute HCl solution free from protein, under suitable experimental conditions an osmotic equilbrium is reached after about 6 hours. When at that time the hydrogen ion concentrations of the protein solution and the outside solution are measured, it is found that the hydrogen ion concentration of the outside solution is higher than that of the protein solution, while the chlorine ion concentration is higher in the protein solution than outside.[1] This unequal concentration of the oppositely charged H and Cl ions inside and outside leads to a membrane potential and modifies the osmotic pressure of the protein solution. The values of both effects can be calculated from the differences in the pH (or pCl) inside and outside on the basis of Donnan's equation for membrane equilibria. The agreement between observed and calculated values on the basis of Donnan's equilibrium equation is excellent for the membrane potentials and equally good for the osmotic pressure, except that a slight difference in pH (in the second decimal) has a much greater influence on the calculated values of osmotic pressure than of the P.D. These facts were based on the writer's observations on solutions of gelatin and crystalline egg albumin[1] and they were confirmed by Hitchcock's observations on solutions of edestin.[2]

The writer has shown that if in a solution of gelatin chloride of a certain pH, part of the gelatin in solution is replaced by the

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922; *J. Gen. Physiol.*, 1920–21, iii, 667, 691.

[2] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

same weight of powdered gelatin (without changing the pH) the osmotic pressure of the solution is lowered, and this lowering of the osmotic pressure increases the more the more of the dissolved gelatin is replaced by powdered gelatin.[3] This shows that only that part of the protein which is in true solution, *i.e.* which does not exist in the form of larger aggregates, influences the osmotic pressure of a solution. The question arose whether or not powdered particles of gelatin would influence the membrane potential. To obtain an answer to this question the following experiment was made.

Powdered gelatin going through the meshes of sieve 30 but not through 60 was rendered isoelectric in the way previously described. Part of this isolectric gelatin was melted and the melted and powdered isoelectric gelatin were mixed. The total weight of isoelectric gelatin in 100 cc. solution was always the same, but the proportion of powdered to dissolved gelatin varied as indicated in Table I. Thus when the weight of the powdered gelatin was 0.5 gm., the weight of the dissolved gelatin was about 0.3 gm.; when the weight of the powdered gelatin was 0.2 gm., that of the dissolved was 0.6 gm., etc. 100 cc. of the mixture contained 8 cc. of 0.1 N HCl, and the pH of the gelatin solution (at the equilibrium condition to be described) was between 3.2 and 3.3. At this pH the osmotic pressure of a gelatin solution is nearly a maximum. A 1 per cent solution of gelatin chloride has an osmotic pressure of about 450 mm. water at pH 3.4. Only a small part of this osmotic pressure is due to the osmotic pressure of the protein particles themselves; the rest of the observed osmotic pressure of gelatin chloride solutions of pH 3.4 is due to the excess concentration of the crystalloidal ions inside the collodion bag (in which the osmotic pressure of the gelatin solution is measured) over that of the outside aqueous solution free from gelatin, and this quantity is determined by the Donnan equilibrium.[4]

Collodion bags of a content of about 50 cc. were filled with these suspensions and closed with rubber stoppers perforated with glass tubes serving as manometers to measure the osmotic pressure.[4]

[3] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 232; *J. Gen. Physiol.*, 1921–22, iv, 97.

[4] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 169; *J. Gen. Physiol.*, 1920–21, iii, 691.

The bags were put over night at 21°C. into beakers containing each 350 cc. of 0.001 N HCl in water. The next day the osmotic pressure was read, the P.D. between the gelatin chloride solution and the outside aqueous solution free from gelatin was measured (with a Compton electrometer and indifferent saturated KCl-calomel electrodes), and the pH inside and outside was determined with the hydrogen electrode. Table I gives the results of these observations.

TABLE I.

Influence of Substitution of Powdered for Dissolved Gelatin on Osmotic Pressure and P. D.

Powdered gelatin per 100 cc., *gm*	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Dissolved gelatin per 100 cc., *gm*	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Osmotic pressure	85	132	181	230	268	310	342	406	398
pH inside	3.16	3.20	3.18	3.19	3.22	3.27	3.28	3.30	3.33
pH outside	2.82	2.85	2.85	2.83	2.83	2.85	2.82	2.84	2.87
pH inside minus pH outside	0.34	0.35	0.33	0.36	0.39	0.42	0.46	0.46	0.46
P.D. calculated, *millivolts*	20.0	20.5	19.0	21.0	22.5	24.5	26.5	26.5	26.5
Membrane potentials observed, *millivolts*	Between 23.0 and 18.0	Between 22.0 and 18.0.	23.0	21.0	22.5	25.0	26.0	26.5	27.0
	No constant reading.								

The table confirms the writer's former observation that the osmotic pressure of the gelatin solution diminishes the more the more of the dissolved gelatin is replaced by powdered gelatin. The latter obviously does not participate in the osmotic pressure.

The table shows furthermore that the P.D. observed at equilibrium between the gelatin solution and the outside aqueous solution varies much less than the osmotic pressure. It became necessary to ascertain whether or not this P.D. across the membrane which was measured with the aid of two indifferent electrodes (saturated KCl-calomel solution) was actually determined by the difference in the

hydrogen ion concentrations inside and outside, as we should expect if the membrane potentials are due to a membrane equilibrium. The pH inside and outside was therefore measured with the aid of the hydrogen electrode and the value 58 (pH inside minus pH outside) millivolts is called the calculated P.D. The reader will notice that the difference between the observed membrane potential (measured with indifferent electrodes) and the calculated P.D. is not more than 0.5 millivolt. This leaves no doubt that the observed P.D. is determined by the difference in the hydrogen ion concentration on the opposite sides of the collodion membrane and that this P.D. obeys Donnan's equilibrium equation.[5]

These facts then show that the protein aggregates participate in the Donnan equilibrium almost to the same extent as do the isolated molecules or ions of gelatin, and this participation finds expression in the fact that the membrane potentials are lowered comparatively little when dissolved gelatin is replaced by powdered gelatin. The same particles, however, do not contribute to the osmotic pressure for the reason that their share in the excess of chlorine ions is contained inside the solid particles, where it serves to increase the swelling of the particles. The swelling of solid protein particles is, as Procter and Wilson have shown,[6] due to the increase of osmotic pressure inside the particles caused by the Donnan equilibrium. In our experiment there exists inside of each particle of powdered gelatin a Donnan equilibrium whereby the concentration of Cl ions inside is greater than outside and this causes an osmotic pressure. Water will, therefore, diffuse into each granule until the cohesion pressure of the solid particles of gelatin equals the osmotic pressure inside the particles due to the Donnan equilibrium, and the particles will swell. When we therefore have a mixture of dissolved gelatin and powdered particles (micellæ) we have two different osmotic pressures; namely, first, the osmotic pressure of the gelatin in true solution, and, second, the osmotic pressure inside each solid particle of gelatin. The former is measured by the hydrostatic pressure of the column of water required to equalize the rate of dif-

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920–21, iii, 557, 667; 1921–22, iv, 351. 463.

[6] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

fusion in opposite directions through the membrane. This is the osmotic pressure of the protein solution in Table I. The osmotic pressure inside each particle of solid powdered gelatin results in swelling, *i.e.* in an increase of the force of cohesion between the molecules of the gel particle, and this effect does not appear in the osmotic pressure of the solution. Only that part of the osmotic forces in a protein solution appears in the form of hydrostatic pressure which is directly or indirectly due to the isolated molecules of the protein; and this hydrostatic pressure is diminished when part of the protein in solution is replaced by aggregates or micellæ of protein.

II.

When a solution of gelatin chloride containing solid granules of gelatin is separated by a collodion membrane from an aqueous solution (free from protein) two different equilibria are established; one across the membrane between the aqueous solution outside and the gelatin solution *inside* the membrane, and a second one between the solid granules of gelatin and the gelatin solution in which the granules are suspended. At first thought it might seem strange that when solid granules of isoelectric gelatin are suspended in a solution of gelatin and HCl, there should arise a difference in the distribution of the H and Cl ions inside the solid granules and the surrounding gelatin solution. Yet this is the case, as Table II shows, and the reason is easily understood. In the solid granules of gelatin the concentration of protein molecules is much higher than in the weak solutions of gelatin surrounding the granules, and if HCl is added the concentration of gelatin ions must be higher inside the solid gelatin granules than in the dilute gelatin solution in which the granules are suspended. It follows from Donnan's theory that this difference in the concentration of protein ions inside the powdered particles and the solution must give rise to a Donnan equilibrium; as a consequence of which a P.D. must exist between the solid particles and the weaker gelatin solution.[7]

This consequence of the theory was confirmed by the following experiment. Mixtures of a solution of isoelectric gelatin and

[7] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 145.

powdered isoelectric gelatin were made so that 100 cc. always contained 0.8 gm. of isoelectric gelatin in all. The proportion of solid and liquid gelatin varied, however, in each case as indicated in Table II. In each 100 cc. of the mixture were contained 8 cc. of 0.1 N HCl. The mixtures were kept for 2 hours at 20°C. and frequently agitated to accelerate establishment of equilibrium between granules and solution. The solid powdered gelatin was then separated from the supernatant liquid by filtration.

TABLE II.

Donnan Equilibrium between Particles of Powdered Gelatin and Gelatin in Solution.

Powdered gelatin per 100 cc., *gm*	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Dissolved gelatin per 100 cc., *gm*	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
pH of powdered gelatin		3.30	3.26	3.28	3.24	3.28	3.24	3.30	3.26
pH of supernatant gelatin solution	2.99	2.97	2.90	2.88	2.83	2.77	2.72	2.69	2.62
pH solid minus pH liquid gelatin		0.33	0.36	0.40	0.41	0.51	0.52	0.61	0.64
P.D. calculated, *millivolts*		19.0	21.0	23.0	24.0	29.5	30.0	35.5	37.0
P.D. observed, *millivolts*		14.5	17.0	17.0	17.5	23.0	26.0	30.0	33.0

The solid gelatin was melted and poured into the vessels described on page 154 of a recent book by the writer, and the P.D. between the solid and liquid gelatin was measured. The values are found in Table II, showing that there exists a considerable P.D. between the gelatin granules and weak solutions of gelatin, and that this P.D. increases with the relative increase in the concentration of solid gelatin, as was to be expected.

Measurements of the pH in the solid and liquid gelatin showed that this P.D. was determined by the Donnan equilibrium.[8] There exists, however, a discrepancy between calculated and observed values for the P.D., which requires further investigation. On the whole, however, the figures seem to prove that when a suspension of powdered protein in a weak gelatin solution is put inside a collodion bag, the latter dipped into an aqueous solution free from gelatin, two equilibria are established; namely, one between the solid

[8] The measurements of the P.D. between solid gelatin and solution are not as accurate as the measurements between liquids across a membrane.

gelatin and the solution of gelatin inside the collodion bag, and a second one between the gelatin solution inside the collodion bag and the outside aqueous solution free from gelatin.

This explains why the powdered particles of protein contained in a gelatin solution participate in the Donnan equilibrium and the membrane potential of the solution without adding to the osmotic pressure of the solution as measured by the hydrostatic pressure required to equalize the rate of diffusion of water in opposite directions through the membrane. The participation of the solid particles in the Donnan equilibrium leads to an osmotic pressure inside of each solid granule, but this osmotic pressure is measured in terms of cohesion pressure of the swollen particles.

This swelling of the solid particles increases the viscosity of the solution, and the writer has shown that this fact is a further support of the explanation of colloidal behavior on the basis of the theory of membrane equilibria.[3]

SUMMARY AND CONCLUSIONS.

1. It is shown that when part of the gelatin in a solution of gelatin chloride is replaced by particles of powdered gelatin (without change of pH) the membrane potential of the solution is influenced comparatively little.

2. A measurement of the hydrogen ion concentration of the gelatin chloride solution and the outside aqueous solution with which the gelatin solution is in osmotic equilibrium, shows that the membrane potential can be calculated from this difference of hydrogen ion concentration with an accuracy of half a millivolt. This proves that the membrane potential is due to the establishment of a membrane equilibrium and that the powdered particles participate in this membrane equilibrium.

3. It is shown that a Donnan equilibrium is established between powdered particles of gelatin chloride and not too strong a solution of gelatin chloride. This is due to the fact that the powdered gelatin particles may be considered as a solid solution of gelatin with a higher concentration than that of the weak gelatin solution in which they are suspended. It follows from the theory of membrane

equilibria that this difference in concentration of protein ions must give rise to potential differences between the solid particles and the weaker gelatin solution.

4. The writer had shown previously that when the gelatin in a solution of gelatin chloride is replaced by powdered gelatin (without a change in pH), the osmotic pressure of the solution is lowered the more the more dissolved gelatin is replaced by powdered gelatin. It is therefore obvious that the powdered particles of gelatin do not participate in the osmotic pressure of the solution in spite of the fact that they participate in the establishment of the Donnan equilibrium and in the membrane potentials.

5. This paradoxical phenomenon finds its explanation in the fact that as a consequence of the participation of each particle in the Donnan equilibrium, a special osmotic pressure is set up in each individual particle of powdered gelatin which leads to a swelling of that particle, and this osmotic pressure is measured by the increase in the cohesion pressure of the powdered particles required to balance the osmotic pressure inside each particle.

6. In a mixture of protein in solution and powdered protein (or protein micellæ) we have therefore two kinds of osmotic pressure, the hydrostatic pressure of the protein which is in true solution, and the cohesion pressure of the aggregates. Since only the former is noticeable in the hydrostatic pressure which serves as a measure of the osmotic pressure of a solution, it is clear why the osmotic pressure of a protein solution must be diminished when part of the protein in true solution is replaced by aggregates.

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